JHeimbach LLC

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January 19, 2018

Paulette Gaynor, Ph.D.
Senior Regulatory Project Manager
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Gaynor:

Pursuant to 21 CFR Part 170, Subpart E, Lallemand Health Solutions (Lallemand), through me as its agent, hereby provides notice of a claim that the addition to milk-based term infant formula of three strains of probiotic bacteria (*Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, and *Bifidobacterium bifidum* Rosell®-71), both individually and in an 80:10:10 blend, is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Lallemand has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the GRAS monograph and one signed copy of the statement of the Expert Panel are provided. Additionally, I have enclosed a virus-free CD-ROM with the GRAS monograph and the statement of the Expert Panel.

I apologize for the size of the GRAS monograph, but note that it includes information regarding three bacterial strains that have been extensively studied over many years, both alone and in combination.

If you have any questions regarding this notification, please feel free to contact me at 804-742-5543 or jh@jheimbach.com.

Sincerely,

(b) (6)

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

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LALLEMAND HEALTH SOLUTIONS

Generally Recognized as Safe (GRAS) Determination for the Use of the Probiotics *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), and *Bifidobacterium bifidum* Rosell®-71 (R0071)

Prepared by:
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LALLEMAND HEALTH SOLUTIONS

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PART 1. SIGNED STATEMENTS AND CERTIFICATION

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1.1. GRAS Notice Submission

Lallemand Health Solutions of Mirabel, Québec, Canada (Lallemand) submits this GRAS notification through its agent James T. Heimbach, president of the consulting firm JHeimbach LLC, in accordance with the requirements of 21 CFR Part 170, Subpart E.

1.2. Name and Address of Notifier

Lallemand Health Solutions
17975 rue des Gouverneurs
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Tel (450) 433-9139

Notifier Contact:
Solange Henoud – Regulatory Affairs Director
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Agent Contact:
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1.3. Name of Notified Organisms

The subjects of this Generally Recognized as Safe (GRAS) notification are each of:

- *Lactobacillus helveticus* Rosell®-52 (designated as R0052);
- *Bifidobacterium longum* ssp. *infantis* Rosell®-33 *(*designated as R0033);
- *Bifidobacterium bifidum* Rosell®-71 (designated as R0071); and
- A blend of the three above strains at a respective ratio of 80:10:10

Lactobacillus helveticus Rosell®-52 (R0052) is deposited under number I-1722 at the Pasteur Institute, in the *Collection Nationale de Cultures de Microorganismes* (CNCM).

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033) *is* deposited under number I-3424 at the Pasteur Institute, in the CNCM.

Bifidobacterium bifidum Rosell®-71 (R0071) is deposited under number I-3426 at the Pasteur Institute, in the CNCM.

1.4. Intended Conditions of Use

A blend of *Lactobacillus helveticus* Rosell®-52 (80%), *Bifodobacterium longum* ssp. *infantis* Rosell®-33 (10%), and *Bifodobacterium bifidum* Rosell®-71 (10%), is intended to be added to powdered milk-based infant formula intended for healthy term infants. The intended addition level is $5x10^7$ cfu/g powder in formulas with hydration rates of 12.5-13.5 g/100 ml, resulting in an initial load of $5x10^9$ cfu/800 ml hydrated formula, designed to result in intake of at least $3x10^9$ cfu per day throughout the shelf life of the formula, allowing for some loss of viability.

This GRAS notification also provides for the addition of each strain individually (i.e., to be added alone to powdered infant formula), at a similar level, resulting under the same conditions to intakes as follows:

- *Lactobacillus helveticus* Rosell®-52: up to $3x10^9$ cfu/day.
- *Bifodobacterium infantis* Rosell®-33: up to $3x10^9$ cfu/day.
- *Bifodobacterium bifidum* Rosell®-71: up to $3x10^9$ cfu/day.

1.5. Statutory Basis for GRAS Status

Lallemand Health Solution's GRAS determination for the intended use of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), and *Bifidobacterium bifidum* Rosell®-71 (R0071), individually or as an 80:10:10 blend, is based on scientific procedures as described under 21 CFR §170.30(b).

1.6. Premarket Exempt Status

The intended use of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), and *Bifidobacterium bifidum* Rosell®-71 (R0071), individually or in an 80:10:10 blend, is not subject to the premarket approval requirements of the Federal Food Drug and Cosmetic Act based on Lallemand's conclusion that such use is GRAS.

1.7. Availability of Information

The data and information that serve as the basis for the GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHeimbach LLC, 923 Water Street, P.O. Box 66, Port Royal, Virginia 22535, telephone 804-742-5543 and e-mail jh@jheimbach.com.

1.8. Freedom of Information Act Statement

None of the information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.9. Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information as well as favorable information known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), and *Bifidobacterium bifidum* Rosell®-71 (R0071), individually or in an 80:10:10 blend.

1.10. FSIS Statement

Not applicable.

1.11. Name and Title of Signer

(b) (6)

James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to Lallemand Health Solutions

PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND TECHNICAL EFFECT

Sections:

2.1. Names of the GRAS Organisms

The subjects of this GRAS notification are:

- *Lactobacillus helveticus* Rosell®-52 (R0052),
- *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033),
- *Bifidobacterium bifidum* Rosell®-71 (R0071), and
- The combination of the three strains at a respective ratio of 80:10:10.

2.2. Sources of the GRAS Organisms

Lactobacillus helveticus Rosell®-52 (R0052) was isolated from a North American dairy starter culture in 1990 by Institut Rosell. The strain has been registered as I-1722 at the Pasteur Institute in the *Collection Nationale de Cultures de Microorganismes* (CNCM).

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033) was isolated by G. Reuter from the intestine of a healthy infant. The strain has been registered as I-3424 at the Pasteur Institute in the CNCM.

Bifidobacterium bifidum Rosell®-71 (R0071) was isolated from the intestine of a healthy adult by G. Reuter. The strain has been registered as I-3426 at the Pasteur Institute in the CNCM.

2.3. Descriptions of the GRAS Organisms

Strains of *Bifidobacterium* and *Lactobacillus* are the most important organisms for human probiotics (O'Sullivan et al. 1992; Fuller and Gibson 1997). Probiotic *Lactobacilli* and *Bifidobacteria* have been used in food products and dietary supplements for decades, with a compelling record of safe consumption (Reid 2002; Kocian et al. 1994; Guidelines FAO/WHO 2002). The organisms that are the subject of this GRAS notice are three thoroughly characterized strains belonging to these genera, as well as a blend

of the three strains that has been safely sold for decades in many countries around the world.

2.3.1. *Lactobacillus helveticus* Rosell®-52 (R0052)

Lactobacillus helveticus was first described by Orla-Jensen in 1919 (Naser et al. 2006). It can be isolated from sour milk and cheese, particularly Emmental and Gruyère cheese (Bergey's Manual of Systematic bacteriology 1986). It is a lactic acid bacterium (LAB) used for centuries in fermented food and has been well studied. Strain Rosell®-52 is a proprietary culture provided to Lallemand Health Solutions (formerly known as Institut Rosell) in 1990 by Weinstein Nutritional Products of California, USA.

The *Lactobacillus* genus appears in the partial list of microorganism compiled by the Food and Drug Administration in 2001. It is provided as an example of harmless LAB that have been prior sanctioned, which ensures FDA viewed the genus as safe (FDA, Partial list of microorganisms 2001).

The International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA) assembled a list of microorganisms with a documented history of safe use in food (Bourdichon et al. 2012). The species *Lactobacillus helveticus* is listed on this inventory. Since 2007, *Lactobacillus helveticus* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authorities (EFSA Journal 2017). A strain belonging to a species listed on QPS and meeting the established criteria can freely be used in foods in Europe.

In Canada, the *Natural Health Products Regulations* of 2004 classified probiotics under the definition of Natural Health Products. On its probiotics monograph, the Natural and Non-prescription Health Products Directorate (NNHPD) of Health Canada listed *Lactobacillus helveticus* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible to generic structure/function claims in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009). This list included the *acidophilus* species but did not include *L. helveticus*. However, after assessment of the characterization profile of the specific strain R0052, *L. helveticus* R0052 received a letter of non-objection to be used in foods and consequently benefit from the applicable regulatory provisions (letter in Appendix II).

Similarly, the specific strain *Lactobacillus helveticus* R0052 has been approved for use in functional foods in Brazil (RESOLUÇÃO - RE N° 2.123, DE 30 DE MAIO DE 2014), as well as in Australia where the TGA (Therapeutic Goods Administration) listed it on its website as an equivalent to *L. acidophilus* R0052 after approving its taxonomic change (Appendix III).

L. helveticus is also included in the list of "Substances that may typically be considered to be a health supplement" in South Africa (Medicines control council. 2014). Food Safety and Standards Authority of India has recognized *Lactobacillus helveticus* and added it in the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). In Korea, this strain has been referenced in the Health Functional Food Code (2010), to be used in Health Functional Foods.

In China, *Lactobacillus helveticus*, is included in the positive list of strain to be used in foods/health foods (Appendix IV).

As aforementioned, the *Lactobacillus helveticus* R0052 strain was obtained by Lallemand Health Solutions (formerly known as Institut Rosell) in 1990. It is a proprietary culture provided to Institut Rosell by the company Weinstein Nutritional Products of California, USA. *Lactobacillus helveticus* R0052 was isolated in 1990 from a dairy culture. The strain is deposited in the CNCM at Pasteur Institute, which guarantees to have an isolate of the strain in a safe and secure place at all times.

2.3.1.1. Phenotypic Identification of *Lactobacillus helveticus* Rosell®-52 (R0052)

Morphology

- Rods, tend to form pairs or short chains especially in the later logarithmic phase of fermentation (see Figure 1).
- Non-motile.
- Non spore-forming.
- Cell size: 0.6 to 0.9 µm width x 1.5 to 6 µm length.
- Forms small white colonies on selective media (see Figure 2 below).

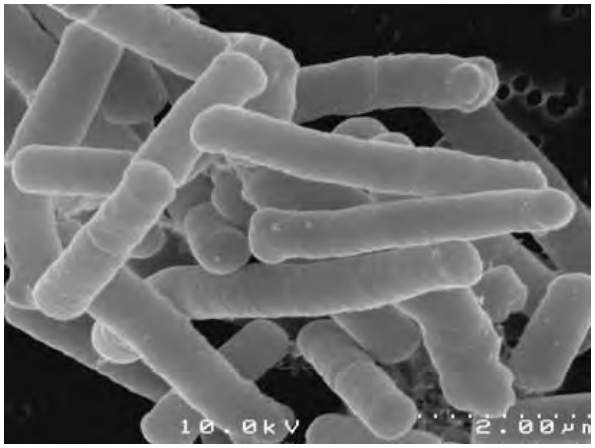

Figure 1. *L. helveticus* Rosell®-52 (R0052) (Magnification 15 000x)
Scanning Electron Micrograph photo by Dr. A. Smith, U. of Guelph, (Ont), Canada.

Figure 2. Colonies of Strain R0052 on MRS Agar.

Gram Stain Reaction

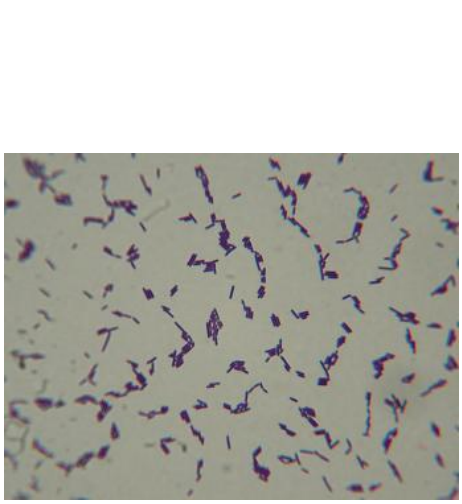

Figure 3. Microscopic Observation of Gram stained *L. helveticus* Rosell®-52 (R0052) (Magnification 1000X).

Biochemical Testing

Fermentative metabolism	Obligately homofermentative Produces mainly lactic acid during fermentation Trace of acetic acid: 0.28 g/L
Gram Stain	+
Catalase (18-24 h colonies on M.R.S. agar, 37°C, anaerobically)	-
β-Galactosidase	+
Lactic Acid type (D/L-lactic acid Kit M.R.S. broth, 16-18h at 37°C, anaerobically)	D:L (9.08 g/L:6.19 g/L)
Optimal Growth Temperature	37ºC
Oxygen requirement	Facultative anaerobe
Hydrogen Peroxide production (Test strip, Phosphate buffer containing glucose, 18-48h at 37°C, aerobically)	Low level 1 mg/L

API Analysis (BioMérieux)

Lactobacillus identification is usually performed by standard testing and by API 50 CHL System (Biomerieux, France), according to Bergey's Manual of Systematic Bacteriology.

Strain R0052 is able to grow on different sugars (see API 50 CHL results).

API 50 CHL (37°C, 48 hours)									
Control	-	Galactose	+	α-methyl-D-mannoside	-	Melibiose	-	D-turanose	-
Glycerol	-	D-glucose	+	α-methyl-D-glucoside	-	Sucrose	+	D-lyxose	-
Erythritol	-	D-fructose	+	N-acetyl-glucosamine	+	Trehalose	+	D-tagatose	-
D-arabinose	-	D-mannose	+	Amygdalin	+	Inulin	-	D-fucose	-
L-arabinose	-	L-sorbose	-	Arbutin	+	Melezitose	-	L-fucose	-
Ribose	-	Rhamnose	-	Esculin	+	D-raffinose	-	D-arabitol	-
D-xylose	-	Dulcitol	-	Salicin	+	Starch	-	L-arabitol	-
L-xylose	-	Inositol	-	Cellobiose	+	Glycogen	-	Gluconate	-
Adonitol	-	Mannitol	-	Maltose	+	Xylitol	-	2-ketogluconate	-
β-methyl-xyloside	-	Sorbitol	-	Lactose	+	β-gentiobiose	+	5-ketogluconate	-

2.3.1.2. Genotypic identification of *Lactobacillus helveticus* Rosell®-52 (R0052)

Historically, R0052 was identified as *Lactobacillus acidophilus*. Its phenotypic characteristics and some metabolic activity such as its capacity to produce D-lactate are in accordance with this designation. R0052 was deposited at the Pasteur Institute in the CNCM, where it was originally identified *L. acidophilus*.

Since then, more advanced genetic techniques became available and helped to properly identify microbes, even when very closely related. The DNA amplification, hybridization, and sequencing performed below defined more precisely the identity of the strain R0052. It was demonstrated that R0052 is a *Lactobacillus* member of the *helveticus* species, which is member of the *L. acidophilus* group.

16S rDNA Sequence

The sequence of the 16S rDNA has been used to identify and taxonomically group bacteria and has become standard practice for identification of new isolates. A partial 16S rDNA sequence was obtained for strain R0052 at Lallemand Health Solutions R&D laboratory.

The sequence shows the closest relationship to *Lactobacillus suntoryeus* and *L. helveticus. Lactobacillus suntoryeus* has been ultimately re-classified as *Lactobacillus helveticus* (Naser et al. 2006).

Multi-Locus Sequence Typing (MLST)

To confirm and refine the taxonomy of this bacterial strain, sequence data of several housekeeping genes were obtained and used to evaluate the relatedness between

strain R0052 and some *L. helveticus* strains (Naser et al. 2006). The partial sequences for the following genes were obtained:

- the gene encoding the alpha subunit of ATP synthase (atpA),
- the gene encoding the RNA polymerase alpha subunit (rpoA),
- the gene encoding the phenylalanyl-tRNA synthase alpha subunit (pheS).

The following reference strains were used (^{T}: type strain):

- *Lactobacillus suntoryeus* LMG 22464^T
- *Lactobacillus suntoryeus* LMG 22465
- *Lactobacillus helveticus* LMG 6413^T
- *Lactobacillus helveticus* LMG 13522
- *Lactobacillus helveticus* LMG 11445
- *Lactobacillus helveticus* LMG 11447
- *Lactobacillus gallinarum* LMG 9435^T
- *Lactobacillus helveticus* LMG 18225

Lactobacillus casei LMG 6904T was included as an outgroup. Bootstrap values (≥50) after 500 simulations are shown. Bar, 2 % sequence divergence. (Naser et al. 2006).

Lactobacillus casei LMG 6904T was included as an outgroup. Bootstrap values (≥50 %) after 500 simulations are shown. Bar, 5 % sequence divergence. (Naser et al. 2006).

Lactobacillus casei LMG 6904T was included as an outgroup. Bootstrap values (≥50 %) after 500 simulations are shown. Bar, 5 % sequence divergence. (Naser et al. 2006).

The sequences show high levels of similarity between strain R0052 and both *L. helveticus* and *L. suntoryeus* strains, with more than 99.5% of gene sequence similarities.

Additionally, the translational elongation factor Tu (tuf) and Hsp60 chaperonin (groEL) genes, as well as the 16S-23S rRNA internally transcribed spacer (ITS) were also analyzed, and compared with those of several *L. helveticus* strains.

Lactobacillus casei LMG 6904T was used as outgroup. Bootstrap values (≥50 %) after 500 simulations are shown. Bar, 2 % sequence divergence. (Naser et al. 2006).

A similarity of more than 98.8% was found between strain R0052 and the *L. helveticus* and *L. suntoryeus* strains.

Lactobacillus casei LMG 6904T was used as outgroup. Bootstrap values (≥50 %) after 500 simulations are shown. Bar, 5 % sequence divergence. (Naser et al. 2006)

A similarity of more than 99% was found between the partial sequences of groEL of strain R0052 and of the investigated *L. helveticus* and *L. suntoryeus* strains.

Lactobacillus casei LMG 6904T was used as outgroup. Bootstrap values (≥50 %) after 500 simulations are shown. Bar, 5 % sequence divergence. (Naser et al. 2006)

Strain R0052 showed 100% similarity with both *L. suntoryeus* strains, and respectively 99% and 98.5% similarity with *L. helveticus* strains LMG 6413^T and LMG 11445.

DNA-DNA Hybridization

Finally, Naser et al. (2006) performed DNA-DNA hybridizations between strain R0052, *L. helveticus* strains LMG 6413T and LMG 11445, and *L. suntoryeus* LMG 22464T.

DNA-DNA hybridization is a method whereby genomic DNA from bacteria is extracted, fluorescently labelled, and used as a probe in hybridization with other genomic DNA extracts. These data are used to obtain a value for the overall DNA homology between two genomes, expressed as a percentage. A DNA-DNA homology value of $>$ 70% is considered the minimum limit for species delineation. Hybridization values of 80% were obtained between strain R0052 and type strain *L. helveticus* LMG 6413^T, and between strain R0052 and type strain *L. suntoryeus* LMG 22646^T.

According to Naser et al. (2006) *L. suntoryeus* is equivalent to *L. helveticus*. These results clearly indicate that R0052 is part of the *L. helveticus* specie.

The results on the tables below clearly indicate that R0052 is part of the *L. helveticus* species.

Table 1. DNA homology results for *L. helveticus* R0052, representatives of the *L. helveticus* group and *L. helveticus* (results from Dr. Denis Roy, Centre de Recherche et de Développement sur les Aliments (CRDA), St-Hyacinthe, Québec).

Strain	R0052
R0052	100
L. helveticus ATCC 15009T	**85**
L. acidophilus ATCC 4356T	47
L. amylovorus ATCC 33620T	52
L. crispatus ATCC 33820T	66
L. gallinarum ATCC 33199T	55
L. gasseri ATCC33323T	44
L. johnsonii ATCC 33200T	56

Table 2. DNA homology results for Lactobacillus helveticus Rosell®-52 (R0052), representatives of *L. helveticus*, *L. gallinarum*, *L. suntoryeus* and *L. helveticus* species (Belgian Coordinated Collections of Micro-organisms (BCCM), Belgium).

Strain	R0052	ATCC 33199	ATCC 15009	ATCC 521	LMG 22464	ATCC 4356
R0052	100					
L. gallinarum ATCC 33199T	44	100				
L. helveticus ATCC 15009T	**80**	47	100			
L. helveticus ATCC 521	**78**	46	**90**	100		
*L. suntoryeus** LMG 22464T	**80**	48	**79**	**70**	100	
L. acidophilus ATCC 4356T	26	35	26	21	23	100

* *L. suntoryeus* has been reclassified as *L. helveticus* (Naser et al. 2006)

Table 3. DNA Homology Results for *L. helveticus* R0052, Representatives of *L. helveticus* and *L. suntoryeus* Species (Naser et al. 2006).

Strain	R0052
R0052	100
L. helveticus LMG 6413	**80**
L. helveticus LMG 11445	**80**
*L. suntoryeus** LMG 22664	**80**

* *L. suntoryeus* has been reclassified as *L. helveticus* (Naser et al. 2006)

16S or 23S rDNA Probe

Different DNA/DNA hybridizations were performed using the R0052 DNA and different 16S/23S rDNA probes specific to other *Lactobacillus* species. As shown in Table 4, *L. helveticus* probe annealed to R0052 DNA, but *L. acidophilus* probe did not, nor did any probe for the *L. acidophilus* group confirming the identity of the strain R0052 as a member of the *helveticus* species.

Table 4. Species Specific rDNA Probe Annealing to R0052 DNA.

Strain	R0052
L. helveticus ATCC 15009T	+
L. acidophilus ATCC 4356T	-
L. amylovorus ATCC 33620T	-
L. crispatus ATCC 33820T	-
L. gallinarum ATCC 33199T	-
L. gasseri ATCC33323T	-
L. johnsonii ATCC 33200T	-

Pulse Field Gel Electrophoresis (PFGE)

Unlike conventional electrophoresis using a unidirectional electric field, pulse field electrophoresis can separate very high molecular weight DNA fragments on agar gel. The PFGE applied to genomic DNA previously digested by an enzyme provides a specific DNA profile for a strain. The cut sites by the enzymes in the DNA vary according to the bacterial species. Therefore, enzymes were selected to give the best profile of a given strain.

Pulse field gel electrophoresis (PFGE) analyses were undertaken for the strain *Lactobacillus helveticus* Rosell®-52 *(R0052)*. The results and methods used are presented below.

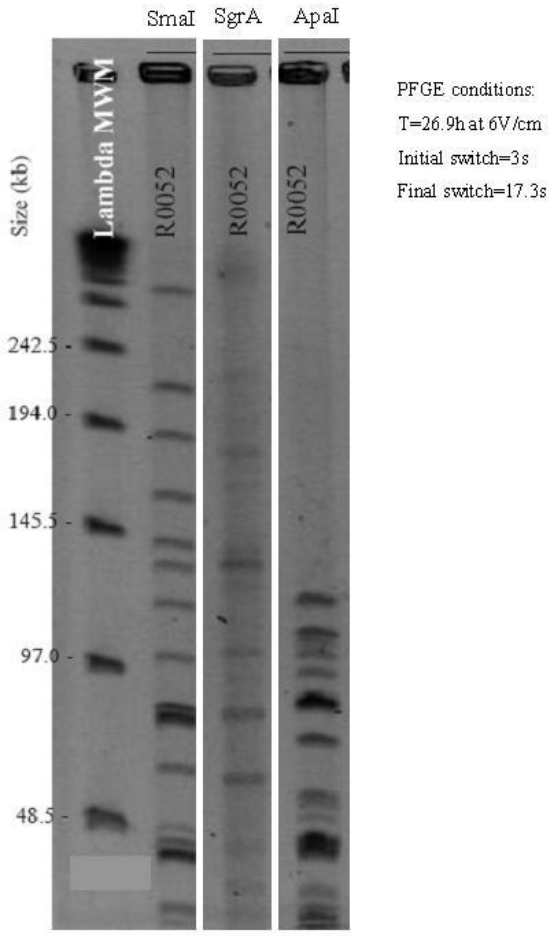

Figure 4. PFGE Profiles of Strain *L. helveticus* R0052 with Enzymes SmaI, SgrAI and ApaI.

Random Amplification of Polymorphic DNA (RAPD)

RAPD is a type of PCR by which the segments of DNA that are amplified are random. The bacterial DNA is extracted, and then amplified using specific primers. RAPD was performed on the strain *Lactobacillus helveticus* Rosell®-52 (R0052) with the following primers: OPA-2, OPA-18, OPL-16, M-14, M-13. RAPD-PCR profiles obtained are shown below.

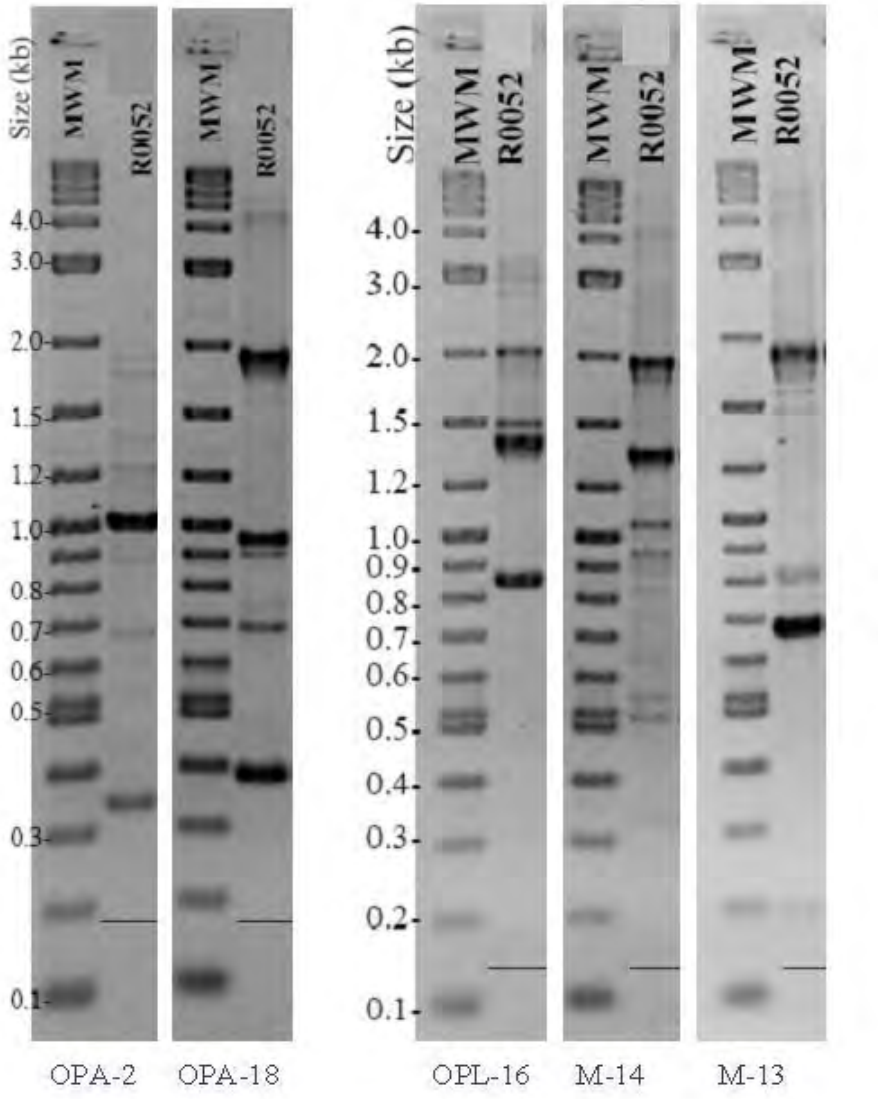

Figure 5. RAPD-PCR Profiles of Strain *L. helveticus* R0052 with Primers OPA-2, OPA-18, OPL-16, M-14, M-13.

2.3.2. *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)

Bifidobacteria predominate in the intestinal tract shortly after birth. They are important and normal constituents of the human gastrointestinal microflora and occur at concentrations of 10^9 to 10^{10} cells/g of feces (Tanaka et al. 2000). *Bifidobacterium infantis* is a natural inhabitant of the intestinal tract microbiota and is a lactic acid bacterium (LAB) which has been used for many years in fermented food.

The International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA) assembled a list of microorganisms with a documented history of safe use in food (Bourdichon et al. 2012). The species *Bifidobacterium infantis* is listed on this inventory. *Bifidobacterium infantis* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authorities (EFSA Journal 2017). A strain belonging to a species listed on QPS and meeting the established criteria can freely be added to foods in Europe.

In Canada, the *Natural Health Products Regulations* of 2004 classified probiotics under the definition of Natural Health Products that can be used to support the general function or structure of the body. This use is in line with the normal intended use of a dietary supplement in the United States. On its probiotics monograph, the Natural and Non-prescription Health Products Directorate (NNHPD) of Health Canada listed *Bifidobacterium longum* ssp. *infantis* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible for generic health claims as well in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009), listing *Bifidobacterium longum* ssp. *infantis* and showing that its use in foods is allowed without a pre-marketing authorization.

The Australian Therapeutic Goods Administration (TGA) includes *B. infantis* on the “List of approved substances that can be used as Active ingredients in “Listed” Medicines”.

Bifidobacterium longum ssp. *infantis* is also included in the list of “Substances that may typically be considered to be a health supplement” in South Africa (Medicines control council. 2014).

Food Safety and Standards Authority of India has also recognized the strain *Bifidobacterium longum* ssp. *infantis* and added it in the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). Additionally, in China, this strains included in the positive list of strain.

2.3.2.1. Phenotypic identification of *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)

Morphology

- Irregular rods, isolated or in short chains (see Figure 6).
- Non-motile.
- Non spore-forming.
- Cell Size: 1 to 1.5 µm width x 4 µm length.
- On selective media *B. longum* ssp. *infantis* R0033 forms small white and smooth colonies (see Figure 7 below).
-

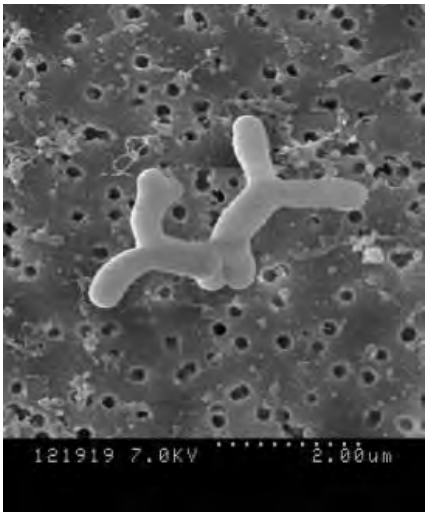

Figure 6. *B. longum* ssp. *infantis* Rosell®-33 (R0033) (Magnification 25,000x) Scanning Electron Micrograph photo by Dr. A. Smith, U. of Guelph, (Ont), Canada.

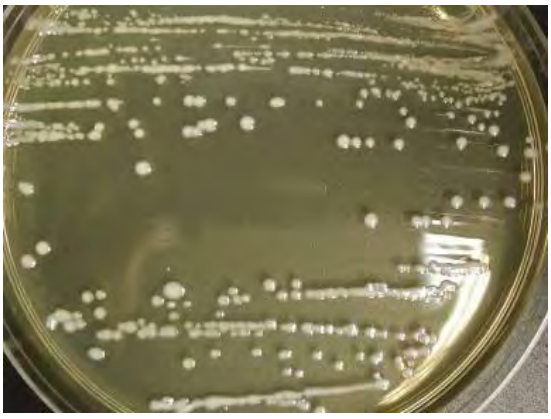

Figure 7. Colonies of *B. longum* ssp. *infantis* Rosell®-33 (R0033) on RCM Agar.

Gram Stain Reaction

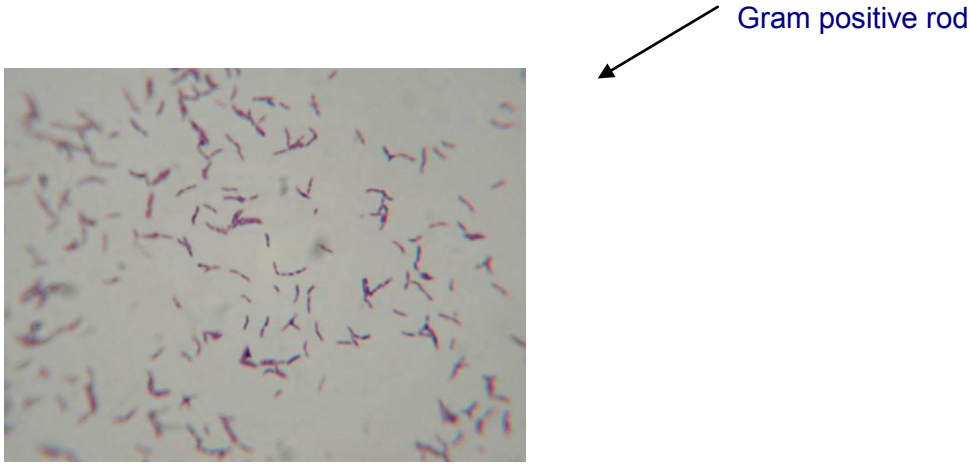

Figure 8. Microscopic observation of Gram stained *B. longum* ssp. *infantis* Rosell®-33 (R0033) (Magnification 1000X).

Biochemical Testing

Heterofermentative	Lactic (2.76 g/L) and acetic (1.92 g/L) acids
Gram Stain	+
Catalase (18-24 h colonies on RCM agar, grown at 37°C anaerobically)	-
Urease (Christensen's urea agar pH 6.8, 6 days at 37°C anaerobically)	+
Lactic Acid type (D/L-lactic acid Kit M30 broth, 16-18 h at 37 °C anaerobically)	L 2.76 g/L
Optimal Growth Temperature	37°C
Oxygen requirement	Strict anaerobe

API Analysis (BioMérieux)

The API 50CHL gallery (BioMérieux) is an identification tool for *Lactobacillus*. It is employed here to characterize the strain *B. longum* ssp. *infantis* R0033 and its metabolic activity, not as a means of identification.

API 50 CHL (37°C, 48 hours)									
Control	-	Galactose	+	α-Methyl-D-mannoside	-	Melibiose	+	D-Turanose	-
Glycerol	-	D-Glucose	+	α-Methyl-D-glucoside	-	Sucrose	+	D-Lyxose	-
Erythritol	-	D-Fructose	+	N-Acetyl-glucosamine	±	Trehalose	-	D-Tagatose	-
D-Arabinose	-	D-Mannose	+	Amygdalin	-	Inulin	-	D-Fucose	-
L-Arabinose	-	L-Sorbose	-	Arbutin	-	Melezitose	-	L-Fucose	±
Ribose	+	Rhamnose	-	Esculin	-	D-Raffinose	+	D-Arabitol	-
D-Xylose	+	Dulcitol	-	Salicin	-	Starch	-	L-Arabitol	-
L-Xylose	-	Inositol	±	Cellobiose	-	Glycogen	-	Gluconate	-
Adonitol	-	Mannitol	-	Maltose	+	Xylitol	-	2-Ketogluconate	-
β-Methyl-xyloside	-	Sorbitol	-	Lactose	+	β-Gentiobiose	-	5-Ketogluconate	-

2.3.2.2. Genotypic identification of *B. longum* ssp. *infantis* Rosell®-33 (R0033)

Whole sequencing of the genome of *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) has been completed. The genotypic characteristics of R0033 have also been determined by the different techniques described hereafter.

16S rDNA Sequence

Partial "16S rDNA" sequence was obtained for *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) by PCR DNA amplification. Two primer sets used to sequence approximately 90% of the 16S rDNA gene were designed: Primer Lb16a, primer 16Smidrev designed *de novo* to pair with Lb16a, primer 16Smidfor modified and now called 16Smidford and finally, primer Lb16b designed *de novo* to pair with 16Smidford.

The sequence shows the closest similarity to the type strain *Bifidobacterium infantis* ATCC 15697. The 16S sequencing confirms the species designation (*B. infantis*) of the strain R0033.

Tuf Gene Sequence

Elongation factor-TU is a protein translation, but has also been found to be exposed on the cell surface of *L. johnsonii* cells and mediates their adherence to Caco-2 cells by interacting mucins (Granato et al. 2004). The *tuf* sequence can be used as a taxonomic tool to identify bacterial isolates in addition to 16S rDNA sequencing.

The sequence shows the closest similarity to the type strain *Bifidobacterium infantis* ATCC 15697. Therefore, the *tuf* gene sequencing also confirms the species designation (*B. infantis*) of the strain R0033.

Pulse Field Gel Electrophoresis (PFGE)

PFGE analyses were undertaken for the strain *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033). The pulse field electrophoresis was applied to genomic DNA, previously digested by an endonuclease, in order to obtain a specific DNA profile for our R0033 strain. Results for each of the two tested enzymes are provided below.

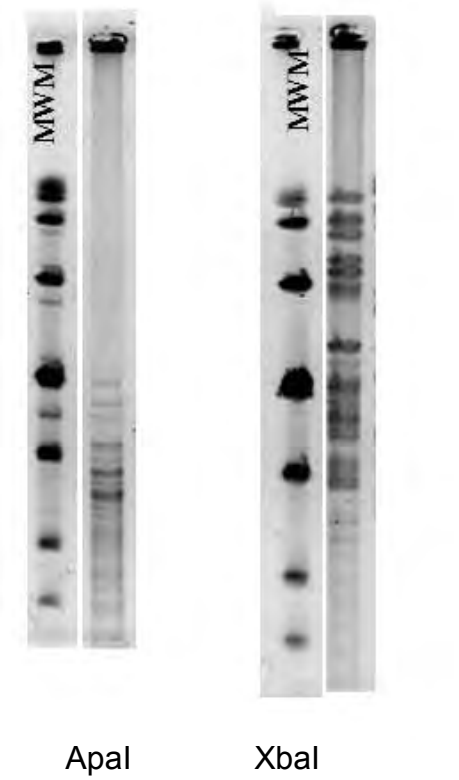

Figure 9. PFGE Profiles for Strain R0033 with Enzymes ApaI and XbaI (10-100 kb).

Random Amplification of Polymorphic DNA (RAPD)

To further characterize the strain R0033, RAPD DNA profiles were obtained using OPA-2, OPA-18, OPL-16, M13 and M14 primers.

The obtained profiles are provided hereafter.

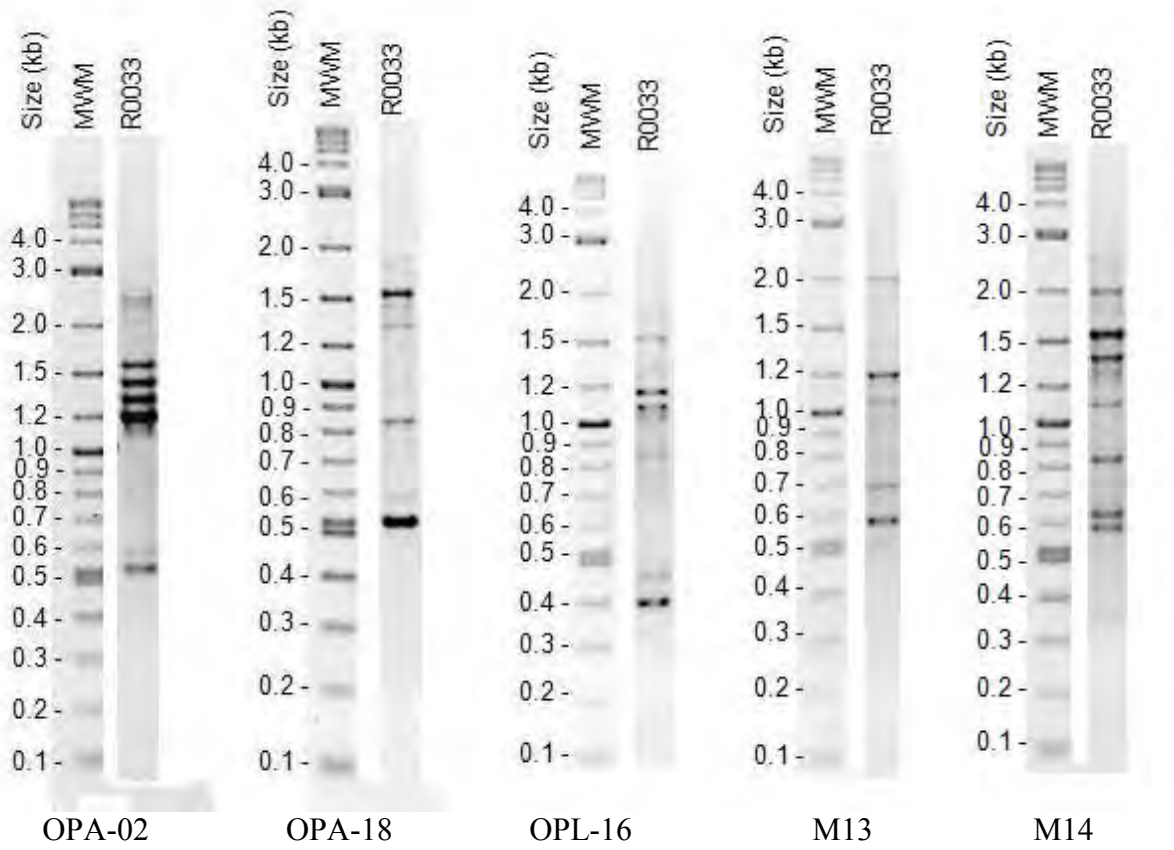

Figure 10. RAPD-PCR Profiles of Strain R0033 with Primers OPA-2, OPA-18, OPL-16, M13 and M14.

2.3.3. *Bifidobacterium bifidum* Rosell®-71 (R0071)

Bifidobacterium bifidum is a natural inhabitant of the intestinal tract microbiota and is a lactic acid bacterium (LAB) which has been used for many years in fermented food.

The International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA) assembled a list of microorganisms with a documented history of safe use in food (Bourdichon et al. 2012). The species *Bifidobacterium bifidum* is listed on this inventory. Since 2007, *Bifidobacterium bifidum* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authorities (EFSA Journal 2017). A strain belonging to a species listed on QPS and meeting the established criteria can freely be added to foods in Europe.

In Canada, the *Natural Health Products Regulations* of 2004 classified probiotics under the definition of Natural Health Products that can be used to support the general function or structure of the body. This use is in line with the normal intended use of a dietary supplement in the United States. On its probiotics monograph, the Natural and Non-prescription Health Products Directorate (NNHPD) of Health Canada listed *Bifidobacterium bifidum* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible to generic health claims as well in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009), listing *Bifidobacterium bifidum* and showing that its use in foods is allowed without a pre-marketing authorization.

The Australian Therapeutic Goods Administration (TGA) includes *B. bifidum* on the “List of approved substances that can be used as Active ingredients in “Listed” Medicines”.

Bifidobacterium bifidum is also included in the list of “Substances that may typically be considered to be a health supplement” in South Africa (Medicines control council. 2014).

Food Safety and Standards Authority of India, has also recognized the strain *Bifidobacterium bifidum* and added it to the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). Additionally, in China, *these both strains are* included in the positive list of strains.

In Korea, *Bifidobacterium bifidum* has been referenced in the Health Functional Food Code (2010), to be used in Heath Functional Foods.

2.3.3.1. Phenotypic identification of *Bifidobacterium bifidum* Rosell®-71 (R0071)

Morphology

- Irregular rods, isolated or in short chains (see Figure 11).
- Non-motile.
- Non spore-forming.
- Cell Size: 1 to 1.5 µm width x 6 µm length.
- On selective media, *B. bifidum* R0071 forms small smooth beige colonies (see Figure 12).

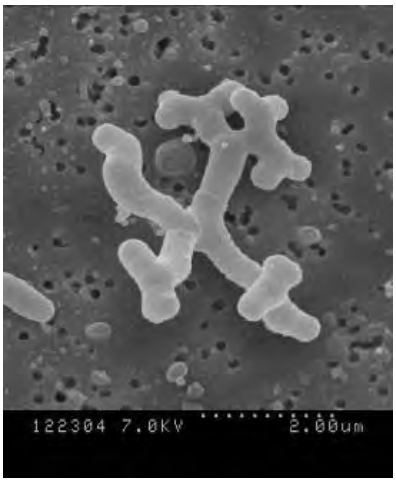

Figure 11. *B. bifidum* Rosell®-71 (R0071) (Magnification 25 000x)
Scanning Electron Micrograph photo by Dr. A. Smith, U. of Guelph, (Ont), Canada.

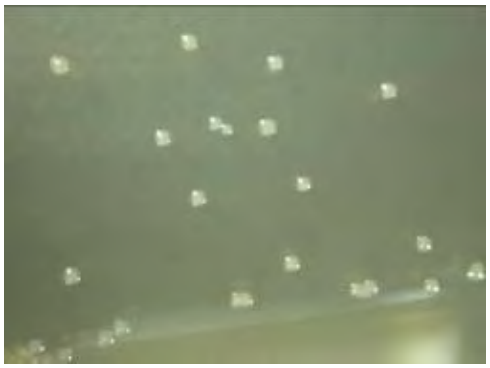

Figure 12. Colonies of *B. bifidum* Rosell®-71 (R0071) on RCM Agar.

Gram Stain Reaction

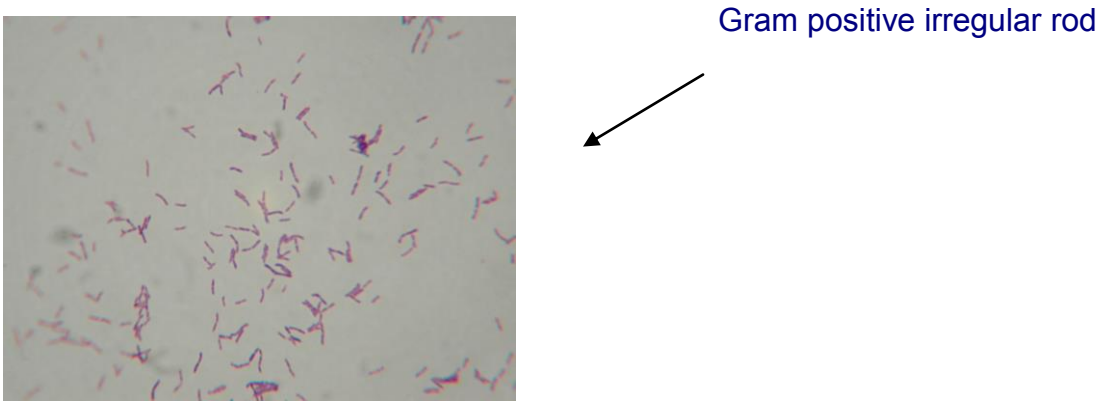

Figure 13. Microscopic Observation of Gram Stained *B. bifidum* Rosell®-71 (R0071) (Magnification 1000X).

Biochemical Testing

Heterofermentative	Lactic (3.7 g/L) and acetic (3.13 g/L) acids
Gram Stain	+
Catalase (18-24 h colonies on RCM agar, grown at 37°C anaerobically)	-
Urease (Christensen's urea agar pH 6.8, 6 days at 37°C anaerobically)	-
Lactic Acid type (D/L-lactic acid Kit M30 broth, 16-18 h at 37 °C anaerobically)	L 3.7 g/L
Optimal Growth Temperature	37°C
Oxygen requirement	Strict anaerobe

API Analysis (BioMérieux)

The API 50CHL gallery (BioMérieux) is an identification tool for *Lactobacillus*. It is employed here to characterize the strain *B. bifidum* R0071 and its metabolic activity, not as a means of identification.

API 50 CHL (37°C, 48 hours)									
Control	-	Galactose	+	α-Methyl-D-mannoside	-	Melibiose	±	D-Turanose	-
Glycerol	-	D-Glucose	+	α-Methyl-D-glucoside	-	Sucrose	-	D-Lyxose	-
Erythritol	-	D-Fructose	+	N-Acetyl-glucosamine	±	Trehalose	-	D-Tagatose	-
D-Arabinose	-	D-Mannose	-	Amygdalin	-	Inulin	-	D-Fucose	-
L-Arabinose	-	L-Sorbose	-	Arbutin	-	Melezitose	-	L-Fucose	-
Ribose	-	Rhamnose	-	Esculin	-	D-Raffinose	-	D-Arabitol	-
D-Xylose	-	Dulcitol	-	Salicin	-	Starch	-	L-Arabitol	-
L-Xylose	-	Inositol	-	Cellobiose	-	Glycogen	-	Gluconate	-
Adonitol	-	Mannitol	-	Maltose	-	Xylitol	-	2-Ketogluconate	-
β-Methyl-xyloside	-	Sorbitol	-	Lactose	+	β-Gentiobiose	-	5-Ketogluconate	-

2.3.3.2. Genotypic identification of *Bifidobacterium bifidum* Rosell®-71 (R0071)

Whole sequencing of the genome of *Bifidobacterium bifidum* Rosell®-71 (R0071) has been recently achieved. The genotypic characteristics of R0071 have also been determined by the different techniques described hereafter.

16S rDNA Sequence

The sequence of the 16S rDNA has been used to identify and taxonomically group bacteria and has become standard practice for identification of new isolates. Partial "16S rDNA" sequence was obtained for *Bifidobacterium bifidum* Rosell®-71 (R0071) by PCR DNA amplification. The partial sequencing was performed by MIDI Labs Inc. (Newark, DE), an external laboratory.

The sequence shows the closest similarity to the type strain *Bifidobacterium bifidum* KCTC 3202. The 16S sequencing confirms the species designation (*B. bifidum*) of the strain R0071.

Tuf Gene Sequence

Elongation factor-TU is a protein translation, but has also been found to be exposed on the cell surface of *L. johnsonii* cells and mediates their adherence to Caco-2 cells by interacting mucins (Granato et al. 2004). The *tuf* sequence can be used as a taxonomical tool to identify bacterial isolates in addition to 16S rDNA sequencing.

The sequence shows the closest similarity to the type strain *Bifidobacterium bifidum* ATCC 29521. Therefore, the *tuf* gene sequencing also confirms the species designation (*B. bifidum*) of the strain R0071.

Pulse Field Gel Electrophoresis (PFGE)

PFGE analyses were undertaken for the strain *Bifidobacterium bifidum* R0071. The pulse field electrophoresis was applied to genomic DNA, previously digested by an endonuclease, in order to obtain a specific DNA profile for our R0071 strain. Results for each of the two tested enzymes are provided below.

Reference: Roy et al. (1996)
R0071 is identified as RW-012 on the paper published by Roy et al. (1996)

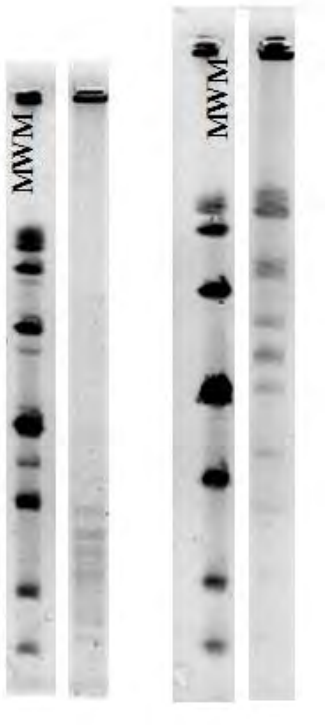

Figure 14. PFGE Profiles for Strain R0071 with Enzymes Apal and Xbal (10-100 kb).

Random Amplification of Polymorphic DNA (RAPD)

To further characterize the strain R0071, RAPD DNA profiles using OPA-2, OPA-18, OPL-16, M13 and M14 primers.

The obtained profiles are provided hereafter.

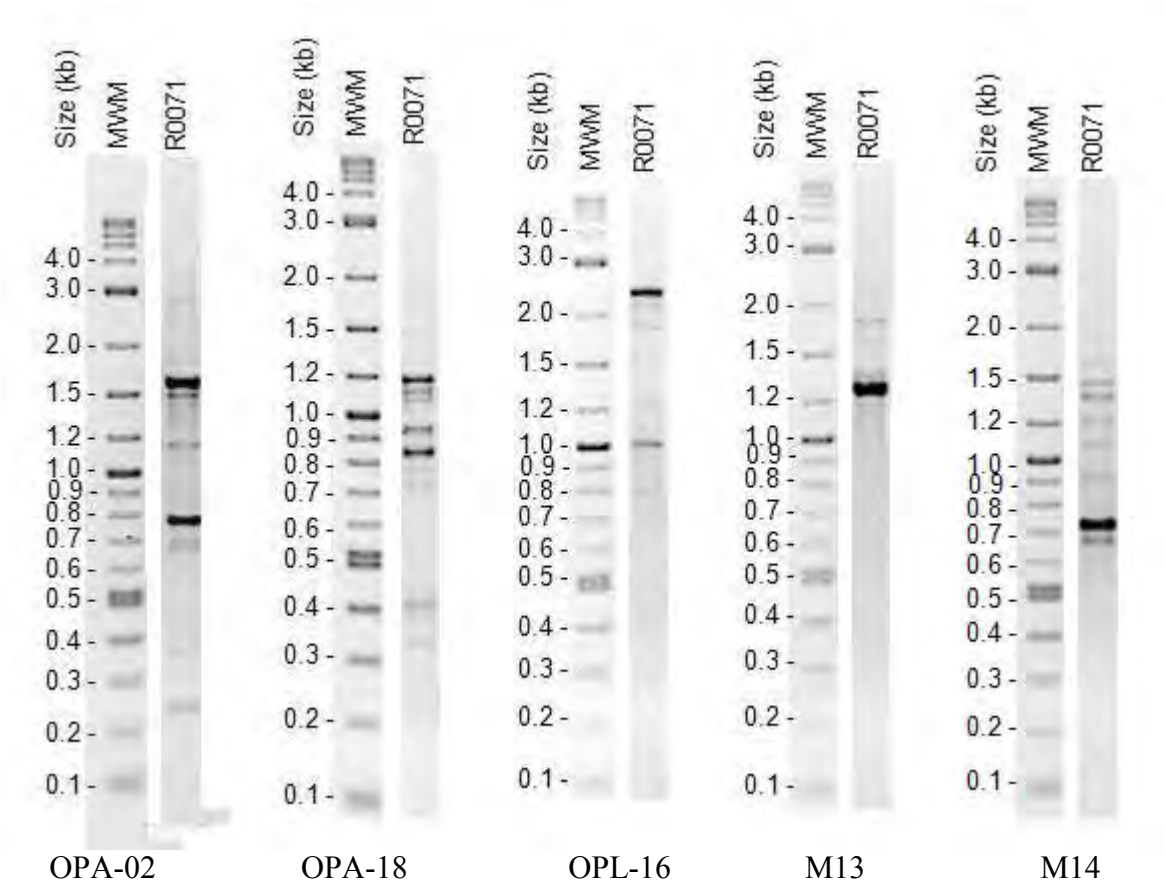

Figure 15. RAPD-PCR Profiles of Strain R0071 with Primers OPA-2, OPA-18, OPL-16, M13 and M14.

2.4. Genomic Analysis

All three bacterial strains have been sequenced and annotated to assure that the strains do not harbor known virulence genes, potentially transferable antibiotic resistance genes, or the capability to synthesize biogenic amines.

2.4.1. *Lactobacillus helveticus Rosell®-52 (R0052)*

2.4.1.1. Sequencing

The whole genome sequence of *Lactobacillus helveticus* strain R0052 was determined and the resulting sequence was annotated and analyzed for genes that may be possible safety concerns. R0052 genomic DNA (gDNA) was sequenced by 454 sequencing technology (Roche, Branford CT). The whole genome sequencing statistics are displayed in Table 5. A final assembly was conducted with a whole-genome optical map to validate the assembly of the single final contig that was 2,129,206 nucleotides long. This genome was deposited in GenBank under the accession number CP003799 and was publicly released with a genome announcement article in 2012 *(*Tompkins TA, et al. 2012).

Table 5. R0052 Genome Sequencing Statistics.

Element	Quantity
Reads	341383
Total coverage	51-fold
Initial assembly contigs	21
Final assembly contigs	1
Genome size (nt)	2129206
GC content (%)	36.8

A phylogenetic tree was built to show the accurate taxonomic identification and the differences between closely related strains.

The *L. helveticus* R0052 clustered within the *L. helveticus* group and the Optical map profile was 91.1% different to the ATCC 15009 type strain.

Another visual analysis of the taxonomy identification was performed, which is a dendrogram based on genomic BLAST and retrieved from the NCBI website (NCBI: https://www.ncbi.nlm.nih.gov/genome). It can be seen that the R0052 strain clustered closely to LMG 22464, which was initially named a *Lactobacillus suntoryeus.* However, based on scientific evidence and a 2006 publication, it was accepted that the *L. suntoryeus* species was grouped with the *L. helveticus* species under the *L. helveticus* name (Naser et al. 2006).

2.4.1.2. Annotation of the Genome

Annotation of the genome R0052 sequence was done online using Rapid Annotation using Subsystem Technology pipeline (RAST; http://rast.nmpdr.org/rast.cgi). The RAST server was developed to annotate microbial genomes. It works by projecting manually curated gene annotations from the SEED database onto newly submitted genomes. The genome annotation statistics are displayed in Table 6. There were 40% (905) open-reading frames (ORFs) or protein encoding genes (PEG) with assigned functions and 60% (1384) ORFs with unknown functions or hypothetical proteins.

Table 6. R0052 Genome Annotation Statistics.

Element	Quantity
ORFs	2289
RNA coding sequences	73
ORFs in subsystem	905
ORFs not in subsystems	1384

2.4.1.3. Annotation of the Plasmid

The *Lactobacillus helveticus* strain R0052 contains a small cryptic plasmid comprised of 8 ORFs. The plasmid was named pIR52-1 and the annotation was performed with the GAMOLA software (Altermann et al. 2003). The plasmid sequence was submitted to the National Center for Biotechnology Information (NCBI) GenBank database under accession number FJ851149. The pIR52-1 plasmid discovery was published (Hagen et al. 2010). This plasmid does not harbor any genes of safety concern as no antibiotic resistance or virulence gene were present among the 8 ORFs.

2.4.1.4. Results of the Genomic Analysis

Antibiotic Resistance

The whole genome sequence was used to screen two antibiotic resistance gene databases. ResFinder v2.1 database is a peer-reviewed and published database that is used for screening of acquired antibiotic resistance (ABR) (Kleinheinz et al. 2014). This validated database uses a homology search tool such as BLAST to screen the input sequences. ResFinder contains more than 2000 ABR genes and is updated periodically. It did not find matches to any ABR genes. In addition, the ARG-ANNOT ABR gene database is downloadable software that can be used to detect existing and putative new antibiotic resistance in bacterial genomes (Gupta et al. 2014). This database also utilizes a BLAST approach for sequence complementary screening of 1689 ABR genes. Screening of the R0052 genome using this database did not reveal any genes of concern.

Synthesis of Biogenic Amines

Microbial biogenic amine formation occurs via the decarboxylation of amino acids that produce metabolites such as histamine, tyramine, cadaverine and putrescine. Therefore, the R0052 strain was analyzed for these four biogenic amines in culture supernatants with High Performance Liquid Chromatography (HPLC). No biogenic amines were detected in the R0052 samples.

The genome was also screened for the presence of amino acid decarboxylases. A single hit was obtained as ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine (a product of the urea cycle from arginine) to form putrescine. Putrescine is a biogenic amine in the polyamine category and this product is involved in the foul odor of putrefying flesh, bad breath, and bacterial vaginosis. However, polyamines, of which putrescine is one of the simplest, appear to be growth factors necessary for cell division. Moreover, ODC is an essential enzyme for cell growth, producing the polyamines necessary to stabilize newly synthesized DNA.

Overall, *L. helveticus* strain R0052 harbors only one gene responsible for the production of biogenic amines through amino acid decarboxylation, which is the ODC gene. However, this is also an essential enzyme for the bacteria and the product of this enzyme, putrescine, is only toxic at very high doses. Since putrescine was not detected in culture supernatant, this is not of any concern.

Adhesion

A specific search for annotated genes related to adhesins or to collagen binding activities did not reveal the presence of such a gene in the R0052 genome. Despite this apparent absence, a gene specific search was conducted by searching an adhesin gene from the NCBI GenBank database. Few hits were recovered in Lactobacillus helveticus strains MTCC 5463 and CNRZ32 (accession # AEYL01000182 and NC021744). These genes are indeed present in R0052 with 98% and 95% identity after a homology search, respectively. In MTCC 5463, this gene is annotated as ABC-type metal-ion transport system, periplasmic component/surface adhesin and in the CNRZ32 genome, the annotation only mentions adhesin. Moreover, it was shown in an *in vitro* adhesion assay that the R0052 has the ability to adhere to HT-29 colonic epithelial cells (IECs) and adhesion is mediated by its surface-layer protein, but it is not invasive.

Virulence/Infectivity

Whole-genome screening for known virulence factors in *E. coli, Enterococcus* spp. and *S. aureus* with the VirulenceFinder v1.5 database (Kleinheinz, et al. 2014) resulted in no

homologous matches for R0052. The annotated *L. helveticus* R0052 whole-genome screening resulted in no hits for virulence factors.

2.4.2. *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)

2.4.2.1. Sequencing

The whole genome sequence of *B. longum* ssp. *infantis* R0033 was determined and the resulting sequence was annotated and analyzed for genes that may be possible safety concerns. R0033 genomic DNA (gDNA) was sequenced by the Yale Center for Genome Analyses (YCGA) at Yale University (New Haven, Connecticut). About 5 µg of intact total gDNA were sent to YCGA where a 10 kb library was prepared prior to WGS that was performed by the Pacific Biosciences (PacBio) sequencing technology. A final assembly was conducted with a whole-genome optical map to validate the assembly into a single final contig. See Table 7 for genome sequencing results.

Table 7. R0033 Genome Sequencing Statistics.

Element	Quantity
Reads	300584
Average read length (nt)	3768
Total coverage	30-160-fold
Initial assembly contigs	10
Final assembly contigs	1
Genome size (nt)	2615717
GC content (%)	59.2

A phylogenetic tree was built to show the accurate taxonomic identification and the differences between closely related strains.

The *B. longum* ssp. *infantis* strain R0033 clustered within the *B. longum* ssp. *infantis* group and the Optical map profile was 67.2% different to the ATCC 15697 type strain.

Based on the phylogenetic analysis, by clustering closer to the *B. longum* ssp. *infantis* rather than other *Bifidobacterium* species, it shows that the identification was appropriately determined.

2.4.2.2. Annotation of the Genome

Annotation of the whole-genome of R0033 sequence was done online using the RAST pipeline (http://rast.nmpdr.org/rast.cgi). The RAST server was developed to annotate microbial genomes. It works by projecting manually curated gene annotations from the SEED database onto newly submitted genomes. The resulting genome included a total of 283 subsystems and other genome annotation statistics are displayed in Table 8.

There were 38% (881) open-reading frames (ORFs) or protein encoding genes (PEG) with assigned functions and 62% (1476) ORFs with unknown functions or hypothetical proteins. RAST predicted that another 6 open-reading frames were “possibly missing.”

Table 8. R0033 Genome Annotation Statistics.

Element	Quantity
ORFs	2357
RNA coding sequences	69
ORFs in subsystem	881
ORFs not in subsystems	1476

2.4.2.3. Annotation of Plasmids

The *Bifidobacterium longum* ssp. *infantis* strain R0033 does not contain any plasmids.

2.4.2.4. Results of the Genomic Analysis

Antibiotic Resistance

The whole genome sequence was used to screen two antibiotic resistance gene databases. First, the ARG-ANNOT ABR gene database is downloadable software that can be used to detect existing and putative new antibiotic resistance in bacterial genomes (Gupta et al. 2014). This database uses a BLAST approach for sequence complementary search. Screening the R0033 genome did not reveal any of the 1689 ABR genes that are included in the database. ResFinder v2.1 database is a peer-reviewed and published database that is used for screening of acquired ABR (Kleinheimz KA et al. 2014). This validated database also uses a homology search tool BLAST to screen the input sequences. ResFinder contains more than 2000 ABR genes and is updated periodically. Screening the R0033 genome only yielded the *fosA* gene that may confer fosfomycin resistance with a 95.76% gene homology. Fosfomycin resistance is common in bacteria.

Synthesis of Biogenic Amines

The R0033 genome was analyzed for genes encoding amino acid decarboxylases that might catalyze the formation of biogenic amines such as histamine, tyramine, cadaverine and putrescine. The only decarboxylase that was related to amino acids was diaminopimelate decarboxylase. This enzyme catalyzes a reaction which produces L-lysine. Overall, *B. longum* ssp. *infantis* R0033 does not harbor any gene responsible for the expression of biogenic amines through amino acid decarboxylation.

Moreover, the R0033 strain was analyzed by HPLC for biogenic amines in culture supernatants. Results showed the absence of biogenic amines in the R0033 supernatant.

Adhesion

Specific search in RAST for annotated genes related to adhesins or to collagen binding activities did not reveal any gene in the R0033 genome. Despite this apparent absence, a gene specific search was conducted by searching for an adhesin gene from the National Center for Biotechnology Information (NCBI) GenBank database. A single hit was recovered in a *Bifidobacterium longum* ssp. *infantis* strain JCM 1222 complete genome (AP010889). This gene is indeed present in R0033 with a 98% identity after homology search but was annotated as "putative truncated adhesin" on NCBI. In other *Bifidobacterium*, this gene is annotated as Fibronectin type III domain. This domain is widely spread in animal species but also found more sporadically in yeast, plant and bacteria proteins. This protein is not related to any case of virulence.

Virulence/Infectivity

The whole genome was screened for known virulence factors in *E. coli*, *Enterococcus* spp. and *S. aureus* with the VirulenceFinder v1.5 database (Gupta et al. 2014). No homologous matches were found in the R0033 genome.

The annotated genome search in RAST resulted in two hits for virulence factors (*mviN* and *vclB*). *MviN* is an essential protein for Murein synthesis in *E. coli.* The whole *vclB* (virulence cluster protein B) sequence (a 1254 nucleotides sequence) was retrieved from NCBI in a *Bifidobacterium longum* strain D2957 (only hit for *vclB* gene search in other strains than *Listeria*) and the full sequence was BLASTed against the R0033 genome. A 95% homology result was obtained on the last 505 nucleotides of the complete gene. This showed that the *vclB* gene is truncated and missing the first 750 nucleotides of the gene sequence, therefore, the gene is not functional.

2.4.3. *Bifidobacterium bifidum* Rosell®-71 (R0071)

2.4.3.1. Sequencing

The whole-genome sequence of *Bifidobacterium bifidum* R0071 was determined and the resulting sequence was annotated and analyzed for genes that may be possible safety concerns. R0071 genomic DNA (gDNA) was sequenced by the Yale Center for Genome Analyses (YCGA) at Yale University (New Haven, Connecticut). About 5 µg of intact total gDNA were sent to YCGA where a 10 kb library was prepared prior to WGS that was performed by Pacific Biosciences (PacBio) sequencing technology. A final assembly was conducted with a whole-genome optical map to validate the assembly of the single final contig. See Table 9 for genome sequencing results.

Table 9. R0071 Genome Sequencing Statistics.

Element	Quantity
Reads	300584
Average read length (nt)	3999
Total coverage	50-fold
Final assembly contigs	1
Genome size (nt)	2320365
GC content (%)	62.6

A phylogenetic tree was built to show the accurate taxonomic identification and the differences between closely related strains.

The *B. bifidum* strain R0071 clustered within the *B. bifidum* group and the Optical map profile was 19.5% different to the ATCC 29521 type strain.

Based on the phylogenetic analysis, by clustering closer to the *B. bifidum* rather than other *Bifidobacterium* species, it shows that the identification was appropriately determined.

2.4.3.2. Annotation of the Genome

Annotation of the whole-genome of R0071 sequence was done online using the RAST pipeline (http://rast.nmpdr.org/rast.cgi). RAST server was developed to annotate microbial genomes. It works by projecting manually curated gene annotations from the SEED database onto newly submitted genomes. The resulting genome included a total of 257 subsystems and other genome annotation statistics are shown in Table 10. There were 41% (782) open-reading frames or protein encoding genes (PEG) with assigned functions and 59% (1171) open-reading frames with putative function or hypothetical proteins with unknown function. RAST predicted that another 7 ORFs were “possibly missing.”

Table 10. R0071 Genome Annotation Statistics.

Element	Quantity
ORFs	1953
RNA coding sequences	64
ORFs in subsystem	782
ORFs not in subsystems	1171

2.4.3.3. Annotation of Plasmids

Bifidobacterium bifidum strain R0071 does not contain any plasmids.

2.4.3.4. Results of the Genomic Analysis

Antibiotic Resistance

The whole-genome sequence was also used to screen two antibiotic resistance gene databases. ResFinder v2.1 database is a peer-reviewed and published database that is used for screening of acquired antibiotic resistance (ABR) (Kleinheinz et al. 2014). This validated database uses a homology search tool such as BLAST to screen the input sequences. ResFinder contains more than 2000 ABR genes and is updated periodically. There were no matches in the R0071 genome to the ABR genes. Additionally, ARG-ANNOT ABR gene database is downloadable software that can be used to detect existing and putative new antibiotic resistance in bacterial genomes (Gupta et al. 2014). This database also utilizes a BLAST approach for sequence complementary search. Screening of the R0071 genome did not reveal any of the 1689 ABR genes that are included in the database.

Synthesis of Biogenic Amines

The R0071 genome was analyzed for genes encoding amino acid decarboxylases that might catalyze the formation of biogenic amines such as histamine, tyramine, cadaverine and putrescine. The only decarboxylase that was related to amino acid metabolism was the Diaminopimelate decarboxylase. This enzyme catalyzes a reaction which produces L-lysine. Overall, *B. bifidum* R0071 does not harbor any gene responsible for the expression of biogenic amines through amino acid decarboxylation.

Moreover, the R0071 cultured supernatant was analyzed by HPLC for biogenic amines. Results showed the absence of the biogenic amines in the R0071 culture samples.

Adhesion

Specific search for annotated genes related to adhesins or to collagen binding activities revealed the presence of a zonadhesin precursor gene in the R0071 genome. This gene, which is found in few *B. bifidum* strains on NCBI, functions in the reproduction metabolism (sperm-egg interactions) in other organism sexual reproduction. Despite this single hit, a gene specific search was conducted by searching an adhesin gene from the National Center for Biotechnology Information (NCBI) GenBank database to retrieve adhesion sequences from *Bifidobacterium*. Two hits were recovered in a *B. bifidum* strain BGN4 complete genome (CP001361). These genes are present in R0071 with a 99% identity after homology search but were annotated as “ABC-type metal ion

transport system, periplasmic/surface adhesin" on NCBI. Therefore, they are zinc-binding and manganese-binding lipoprotein transporters. None of the adhesion genes found in the R0071 genome is related to virulence.

Virulence/Infectivity

The genome of R0071 was screened for known virulence factors in *E. coli*, *Enterococcus* spp. and *S. aureus* with the VirulenceFinder v1.5 database (Kleinheinz et al. 2014). No homologous matches were found in R0071.

The annotated genome search in RAST resulted in two hits for virulence factors (*mviN* and *vclB*). *MviN* is an essential protein for Murein synthesis in *E. coli*. The whole *vclB* (virulence cluster protein B) sequence (a 1254 nucleotides sequence) was retrieved from NCBI in a *B. longum* strain D2957 (only hit for *vclB* gene search in other strains than *Listeria*) and the full sequence was BLASTed against the R0071 genome. An 88% homology result was obtained on the last 315 nucleotides of the complete gene from the D2957 strain. This showed that the *vclB* gene is truncated and missing the first 939 nucleotides, therefore, the gene is not functional.

2.5. Production Process

The manufacturing process presented in the following sections, comprises the production of each lyophilized bacterial strain as a powder (section 2.5.1) followed by blending of the three lyophilized bacterial strains [*Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) *Bifidobacterium bifidum* Rosell®-71 (R0071)] at a respective ratio of 80:10:10.

Information regarding the facilities involved in the manufacture and testing of each lyophilized bacterial strain and their blend, including the responsibilities of each, is provided in Table 11.

Table 11. Facilities and Responsibilities.

Name and Address	Activity
LALLEMAND HEALTH SOLUTIONS INC. (formerly Institut Rosell Inc.) 8480 Saint Laurent Boulevard Montreal, Quebec, H2P 2M6 Canada	Production of Lactic Acid Bacteria: Culture Collection, Fermentation, Concentration, Freeze-Drying, Quality Control, Storage
LALLEMAND S.A.S. 4, Chemin du Bord de l'Eau 15130 Saint Simon France	Production of Lactic Acid Bacteria: Fermentation, Concentration, Freeze-Drying, Quality Control, Storage Production of Blend: Blending, Packing, Quality Control, Storage

The facility LALLEMAND HEALTH SOLUTIONS INC, is compliant with the requirements for cGMP set by the local authority (Health Canada) for the manufacturing and handling of the strains under Part 3 of the *Natural Health Products Regulation* of 2004.

The facility LALLEMAND SAS is a contract manufacturer belonging to the group Lallemand. It conforms to Lallemand Health Solutions Quality standards. The site is located in France and has been successfully inspected by the US FDA for compliance with the Food Safety Modernization Act (FSMA).

2.5.1 Manufacturing Process of the Bacterial Strains

The manufacturing process of the strains *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) , and *Bifidobacterium bifidum* Rosell®-71 (R0071) is carried out through the steps listed and described schematically in Figure 16 at Lallemand Health Solutions (located in Canada) or Lallemand SAS (located in France).

The manufacturing process comprises the following steps:

Revivification of the Bacterial Strain

A frozen cryotube from the production cell bank is thawed and transferred into a test tube containing sterilized culture medium (previously prepared). The culture is incubated without agitation.

Subculture

The revivified bacterial strain is transferred to an Erlenmeyer flask containing sterilized culture medium. The subculture is incubated.

An aliquot is taken at the end of the incubation period to determine the following parameters: pH and absence of contamination.

Seed Culture

An aliquot from the subculture is transferred to a large Erlenmeyer flask containing sterilized culture medium. The seed is incubated. The following parameters are measured: temperature, pH, optical density, and absence of contamination.

Culture Medium Preparation

The raw materials are checked for identity and weighed per culture media recipe. They are then dissolved in water in the fermenter. The pH is adjusted. The culture media is heat treated *in situ* and cooled prior to inoculation with the seed culture. The temperature is continuously monitored during preparation, sterilization, and cool-down.

Fermentation

A pre-fermentation step may be performed in a smaller fermenter prior to the fermentation when larger inoculum volume is required.

The seed culture is transferred from the Erlenmeyer flask to the heat treated culture media for biomass production (“fermentation”). The choice of the fermenter depends on the quantity of biomass required. During the fermentation, the culture is gently agitated and temperature is controlled. The bacterial strain is grown in the fermenter until the late exponential phase.

Sampling of the culture broth is done periodically during the fermentation to verify the following parameters: pH, optical density, conductivity and residual glucose.

At the end of the fermentation, a sample is tested by Quality Control for the following specifications: Count of viable cell concentration of the cultured strain and absence of contaminants.

Concentration – Centrifugation
The fermentation broth is concentrated by high speed centrifugation.

Cryoprotection and Freeze-Drying
Cryoprotectants are blended with the concentrated bacterial culture until a homogenous solution is obtained. Sterile trays are filled with the blend and introduced into the freeze-dryer.

The trays are then freeze-dried. Temperature of the freeze-dryer and of the concentrated culture is monitored throughout the process. The freeze-drying process consists of a primary drying phase under a partial vacuum to sublimate free water and a secondary drying phase under a vacuum to eliminate water linked to the bacteria cells. The freeze-dried bacterial culture cake-like is collected in double bags and stored under refrigerated conditions until grinding.

Grinding and Packaging
The freeze-dried bacterial concentrates are ground and collected in laminated foil bags. The bags are sealed, weighed, labelled and inventoried. They are then placed in covered bins for storage.

A sample of the bacteria powder is brought to Quality Control for determination of viable cell concentration of the cultured strain, absence of contaminants, water activity and physical parameters.

Storage
The freeze-dried ground bacterial cultures are kept in frozen storage.

All components of the growth medium and the cryoprotectant are food grade, pharmacopeial or equivalent standards (see APPENDIX V for details).

A flow chart of the manufacturing process is provided in Figure 16.

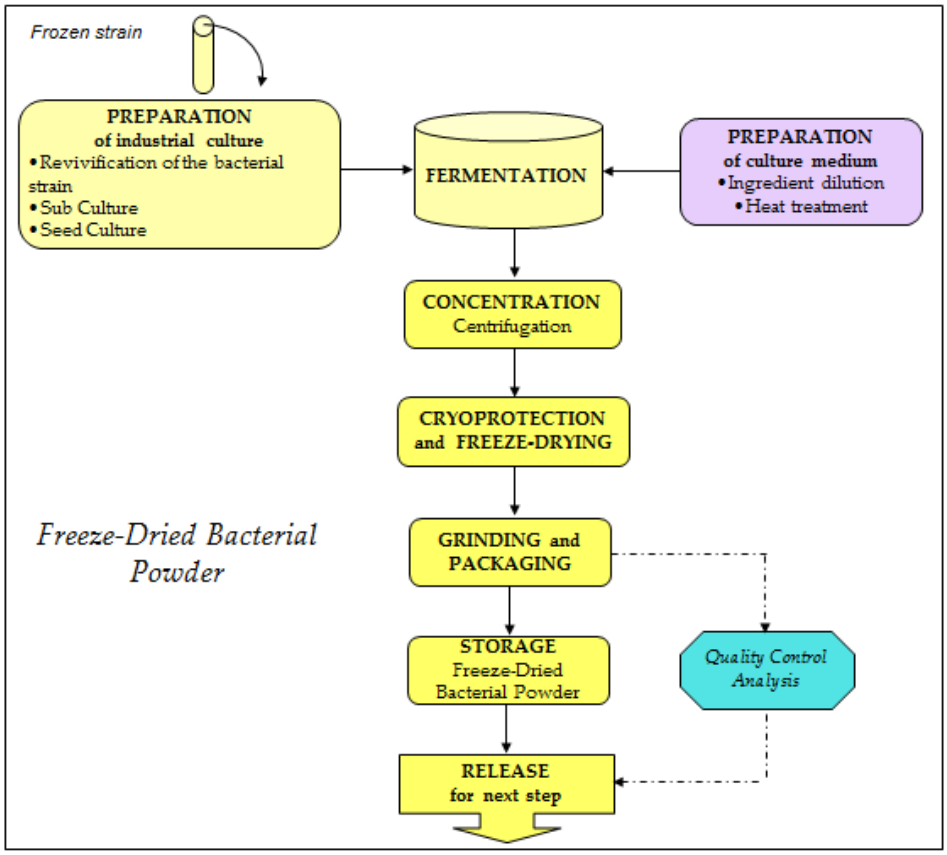

Figure 16. Flow Diagram of Manufacturing Process of the Strains.

2.5.2. Blending of the Three Bacterial Strains

Strains	Quantity per Gram
L. helveticus Rosell®-52 (R0052)	**$4x10^9$ cfu***
B. longum ssp. *infantis* Rosell®-33 (R0033)	**$5x10^8$ cfu***
B. bifidum Rosell®-71 (R0071)	**$5x10^8$ cfu***
Total bacterial culture per gram:	**$5x10^9$ cfu***

A flow diagram for the step of blending is provided in Figure 17.

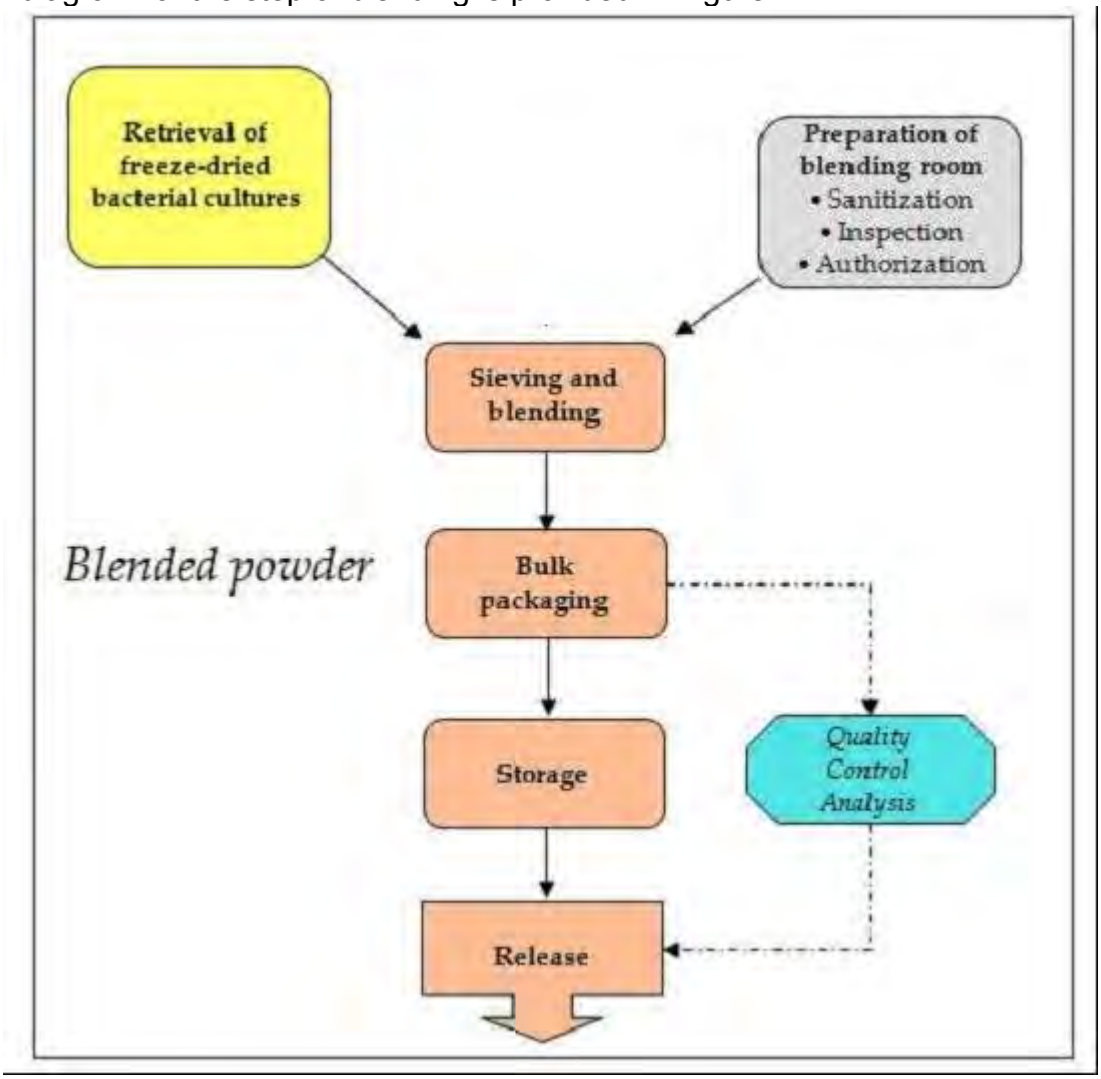

Figure 17. Flow Diagram of Blending Step.

Narrative Description of Blending

Preparation of Freeze-dried Bacterial Cultures

The weighing of freeze-dried bacterial cultures is done in the blending room. The blending room and equipment are cleaned and sanitized before being inspected and authorized for use. The temperature and humidity of the room are monitored and recorded. The freeze-dried bacterial cultures (*Lactobacillus helveticus* Rosell®-52 [R0052], *Bifidobacterium longum* ssp. *infantis* Rosell®-33 [R0033] and *Bifidobacterium bifidum* Rosell®-71 [R0071]) are retrieved and the code and lot numbers are checked against the order form. The bacterial powders are then weighed out as prescribed by the master formula and verified. The remainder of the active bacterial powder is returned to cold storage.

Blending

The blending process is performed immediately after the weighing of the freeze-dried bacterial cultures, which are sifted and added to the blender. Samples for microbiological and physicochemical testing are obtained from the resulting bulk powder. The bulk powder is put into double bags which are tied, placed in closed barrels and labelled. The bulk powder is then stored under refrigerated conditions.

The prescribed microbiological analyses are conducted on the product. If the results meet the specifications, the lot is approved for packing in laminated foil bags.

2.6. Specifications

2.6.1 Specifications of the Bacterial Powder

All batches of *L. helveticus* R0052 meet the specifications set forth in Table 12.

Table 12. Specifications for *L. helveticus* Rosell®-52 (R0052) Freeze-dried Powder.

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Fine to granular, ivory to beige powder	Visual observation
L. helveticus	NA	Bacteriological enumeration – in-house method[1]
Yeast and Molds	<1000 cfu/g	Enumeration on Sabouraud or PDA culture medium + chloramphenicol, after incubation at 20-25°C for 5 to 7 days
Coliforms	<10 cfu/g	ISO 4831
Escherichia coli	<10 cfu/g	ISO 7251
Staphylococcus aureus	<10 cfu/g	ISO 6888-1
Salmonella spp.	Negative in 25g in 60 samples*	ISO 6579
Cronobacter spp.	Negative in 10g in 30 samples*	ISO 22964
*Certificate of Analysis follows.		

[1] The determination of the bacteriological count is done by plate count using strain specific culturing conditions. .

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CERTIFICATE OF ANALYSIS

Product : Lactobacillus helveticus R0052 ND

Lot : 62/17 S3

Date of analysis: 09/11/17

Manufacturing date 10/17

Microbiological control :

21CFR (code of Federal Regulations) - Part 106 Infant Formula Requirements - Section 106.55 (Controls to prevent adulteration from microorganisms)

Tests	Specifications	Results	Methods
Enterobacter sakazakii (cronobacter spp)	30 x Absent/10g	Complies	ISO 22964
Salmonella spp	60 x Absent/25g	Complies	ISO 6579

Saint-Simon, November 30, 2017

(b) (6)

V. BREUIL
Control Lab Supervisor

(b) (6)

All batches of *B. longum* ssp. *infantis* R0033 meet the specifications set forth in Table 13.

Table 13. Specifications for *B. longum* ssp. *infantis* Rosell®-33 (R0033) Freeze-dried Powder.

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Fine to granular, ivory to beige powder	Visual observation
B. longum	NA	Bacteriological enumeration – in-house method[2]
Yeast and Molds	<1000 cfu/g	Enumeration on Sabouraud or PDA culture medium + chloramphenicol, after incubation at 20-25°C for 5 to 7 days
Coliforms	<10 cfu/g	ISO 4831
Escherichia coli	<10 cfu/g	ISO 7251
Staphylococcus aureus	<10 cfu/g	ISO 6888-1
Salmonella spp.	Negative in 25g in 60 samples*	ISO 6579
Cronobacter spp.	Negative in 10g in 30 samples*	ISO 22964
*Certificate of Analysis follows.		

[2] The determination of the bacteriological count is done by plate count using strain specific culturing conditions.

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CERTIFICATE OF ANALYSIS

Product : **Bifidobacterium infantis R0033**

Lot : **93343**

Date of analysis: 08/11/17

Manufacturing date: 06/17

Microbiological control :

21CFR (code of Feceral Regulations) - Part 106 Infant Formula Requirements - Section 106.55 (Controls to prevent adulteration from microorganisms)

Tests	Specifications	Results	Methods
Enterobacter sakazakii (cronobacter spp)	30 x Absent/10g	Complies	ISO 22964
Salmonella spp	60 x Absent/25g	Complies	ISO 6579

Saint-Simon, November 22, 2017

(b) (6)

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All batches of *B. bifidum* R0071 meet the specifications set forth in Table 14.

Table 14. Specifications for *B. bifidum* Rosell®-71 (R0071) Freeze-dried Powder.

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Fine to granular, ivory to beige powder	Visual observation
B. bifidum	NA	Bacteriological enumeration – in-house method[3]
Yeast and Molds	<1000 cfu/g	Enumeration on Sabouraud or PDA culture medium + chloramphenicol, after incubation at 20-25°C for 5 to 7 days
Coliforms	<10 cfu/g	ISO 4831
Escherichia coli	<10 cfu/g	ISO 7251
Staphylococcus aureus	<10 cfu/g	ISO 6888-1
Salmonella spp.	Negative in 25g in 60 samples*	ISO 6579
Cronobacter spp.	Negative in 10g in 30 samples*	ISO 22964
*Certificate of Analysis follows.		

[3] The determination of the bacteriological count is done by plate count using strain specific culturing conditions.

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CERTIFICATE OF ANALYSIS

Product : **Bifidobacterium bifidum R0071**

Lot : 95967

Date of analysis: 08/11/17

Manufacturing date: 09/17

Microbiological control :

21CFR (code of Federal Regulations) - Part 106 Infant Formula Requirements - Section 106.55 (Controls to prevent adulteration from microorganisms)

Tests	Specifications	Results	Methods
Enterobacter sakazakii (cronobacter spp)	**30 x Absent/10g**	Complies	ISO 22964
Salmonella spp	**60 x Absent/25g**	Complies	ISO 6579

Saint-Simon, November 22, 2017

(b) (6)

2.6.2. Specifications of the Blend of the Three Bacterial Strains

Since the three strains are blended in a simple mechanical process with strains meeting the prescribed specifications, no separate specifications or testing are required for the blend.

However, the three strains are also blended in the same proportions [*L. helveticus* Rosell®-52 (R0052*)* (80)%, *B. longum* ssp. *infantis* Rosell®-33 (R0033) (10%), and *B. bifidum* Rosell®-71 (R0071) (10%)] in production of a product called Probiokid®. This product, sold in sachets also containing potato starch, vanilla flavor, and fructooligosaccharides, has been sold internationally since 2002 for administration to infants and young children. Since it includes ingredients in addition to the three probiotic strains, Probiokid® has established specifications and has been tested for *Salmonella* spp. (60 samples) and *Cronobacter* spp. (30 samples)

All batches of the blend of the of *Lactobacillus helveticus* (80%), *Bifodobacterium bifidum* R0071 (10%), and *Bifodobacterium infantis* R0033 (10%) strains meet the specifications set forth in Table 15 for Probiokid®.

Table 15. Specifications for *Probiokid®.*

Test	Acceptance Criterion	Method
Yeasts and molds	<1000 cfu/g	ISO 7954
Coliforms	<10 cfu/g	ISO 4832
Escherichia coli	<1 cfu/g	ISO 7251
Staphylococcus aureus	<10 cfu/g	ISO 6888
Salmonella spp.	Negative in 25g in 60 samples*	ISO 6579
Cronobacter spp.	Negative in 10g in 30 samples*	ISO 22964
*Certificate of Analysis follows.		

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Fax : +33 (0)4.71.47.12.48

CERTIFICATE OF ANALYSIS

Product : PROBIOKID

Lot : 6532347

Date of analysis: 08/11/17

Manufacturing date: 11/17

Microbiological control :

21 CFR (code of Federal Regulations) - Part 106 Infant Formula Requirements - Section 106.55 (Controls to prevent adulteration from microorganisms)

Tests	Specifications	Results	Methods
Enterobacter sakazakii (cronobacter spp)	30 x Absent/10g	Complies	ISO 22964
Salmonella spp	60 x Absent/25g	Complies	ISO 6579

Saint-Simon, November 22, 2017

(b) (6)

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2.7. Heavy Metals

Data analysis of the content of heavy metals in samples of *Lactobacillus helveticus* R0052, *Bifodobacterium bifidum* R0071, and *Bifodobacterium infantis* R0033 confirms that all three strains meet the specifications set forth in Table 16.

Table 16. Heavy Metals Analysis of *L. helveticus* R0052, *B. bifidum* R0071, and *B. longum* ssp. *infantis* R0033.

Test – Heavy Metals	*Lactobacillus helveticus* R0052	*Bifodobacterium bifidum* R0071	*Bifodobacterium infantis* R0033
Lead (mg/kg)	≤0.05	≤0.05	≤0.10
Cadmium (mg/kg)	≤0.020	NA*	NA*
Arsenic (mg/kg)	≤0.30	≤0.05	≤0.10
Mercury (mg/kg)	≤0.005	≤0.05	≤0.10

*NA= Not available

2.8 Stability

For the three strains (L. *helveticus* R0052, *B. bifidum* R0071, and *B. infantis* R0033), 24 month stability studies have been completed at 4°C and 25°C.

- ***Lactobacillus helveticus* Rosell®-52 (R0052)**

According to the results presented hereafter at 4°C and 25°C, *Lactobacillus helveticus* R0052 will maintain over 50% viability for the 24-month shelf life of the product when stored at 4°C.

Table 17. Stability Data for *L. helveticus* Rosell®-52 (R0052) at 4°C.

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	$2.85x10^{11}$	$2.54 x10^{11}$	$2.10 x10^{11}$	$2.03 x10^{11}$	$1.63 x10^{11}$	$1.56 x10^{11}$
Survival rate (%)	100	91	74	69	59	57

Table 18. Stability Data for *L. helveticus* Rosell®-52 (R0052) at 25°C.

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	$2.31x10^{11}$	$1.20 x10^{11}$	$8.75 x10^{11}$	$6.63 x10^{10}$	$4.64 x10^{10}$	$3.27 x10^{10}$
Survival rate (%)	100	52	38	29	19	13

- ***Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)**

According to the results presented hereafter at 4°C and 25°C, *Bifidobacterium longum* ssp. *infantis* R0033 will maintain over 50% of its viability for the 24-months shelf life of the product when stored at 4°C.

Table 19. Stability Data for *B. longum* ssp. *infantis* Rosell®-33 (R0033) at 4°C.

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	$8.84 x10^{10}$	$6.82 x10^{10}$	$6.42 x10^{10}$	$5.71 x10^{10}$	$6.13 x10^{10}$	$5.80 x10^{10}$
Survival rate (%)	100	79	71	64	69	66

Table 20. Stability Data for *B. longum* ssp. *infantis* Rosell®-33 (R0033) at 25°C.

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	8.84×10^{10}	4.05×10^{10}	3.39×10^{10}	2.08×10^{10}	1.86×10^{10}	1.23×10^{10}
Survival rate (%)	100	47	37	23	19	12

- ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

According to the results presented hereafter at 4°C and 25°C, *Bifidobacterium bifidum* R0071 will maintain over 50% of its viability for the 24-months shelf life of the product when stored at 4°C.

Table 21. Stability Data for *B. bifidum* Rosell®-71 (R0071) at 4°C.

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	1.69×10^{11}	1.26×10^{11}	1.12×10^{11}	1.09×10^{11}	1.00×10^{11}	1.15×10^{11}
Survival rate (%)	100	87	79	65	63	57

Table 22. Stability Data for *B. bifidum* Rosell®-71 (R0071) at 25°C.

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	1.69×10^{11}	8.90×10^{10}	6.80×10^{10}	5.30×10^{10}	5.30×10^{10}	4.70×10^{10}
Survival rate (%)	100	58	45	32	26	20

PART 3. DIETARY EXPOSURE (EDI)

The target dietary intake of the blend of the three strains *Lactobacillus helveticus* Rosell®-52, *Bifodobacterium longum* ssp. *infantis* Rosell®-33, and *Bifodobacterium bifidum* Rosell®-71 at a respective ratio of 80:10:10, or of any of the three strains used alone, is $3x10^9$ cfu/day. The probiotic is intended to be added to powdered milk-based infant formula intended for consumption by healthy term infants. In order to provide $3x10^9$ cfu of the probiotic in 800 ml of hydrated formula (an average daily intake), the probiotic must be present in the powder at a concentration of $3x10^7$ cfu/g powder, assuming a hydration rate of 12.5-13.5 g/100 ml. In order to assure that viable probiotic is present at a concentration of at least $3x10^7$ cfu/g powder through its shelf life, it will be introduced at a concentration of $5x10^7$ cfu/g, leading to a maximum potential daily intake of $5x10^9$ cfu.

If the probiotic is added to a formula with a hydration rate different from 12.5-13.5 g/100 ml, the addition concentration will be adjusted as needed to retain the target intake level of $3x10^9$ cfu/day.

According to tables of daily energy intake by formula-fed infants provided by Fomon (1993), the subpopulation of infants with the highest intake per kg body weight is boys age 14–27 days. The mean energy intake by this group is 121.1 kcal/kg bw/day and the 90th percentile is 141.3 kcal/kg bw/day. Among girls, the highest energy intake is found in the same age group, 14–27 days, and is nearly as high as boys: the mean and 90th energy intake percentiles are 117.8 and 138.9 kcal/kg bw/day. Most term infant formulas contain 67.6 kcal/100 ml when ready to consume. Therefore, to obtain 141.3 kcal energy/kg bw, an infant boy must consume 209.0 ml formula/kg bw. To reach her 90th percentile of energy consumption, 138.9 kcal/kg bw/day, an infant girl must consume 205.5 ml formula/kg bw/day. The 90th percentile of formula intake for the two sexes combined is about 207 ml/kg bw/day. This would result in a 90^{th} percentile exposure $8x10^8$ cfu probiotic/kg bw/day, which represents the EDI for the probiotic blend.

When the combination of the three strains is consumed at the stated concentration at ratios of 80:10:10, the EDI of each strain is as follows:

Lactobacillus helveticus Rosell®-52 (R0052)—$6.4x10^8$ cfu/kg bw/day

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033)—$8x10^7$ cfu/kg bw/day

Bifidobacterium bifidum Rosell®-71 (R0071)—$8x10^7$ cfu/kg bw/day

The target population is healthy infants and toddlers aged 0-3 years of age. Since it is not expected that these infants and toddlers will have other dietary sources of any of the three strains of bacteria addressed in this GRAS notice, these figures represent the total EDI from all sources.

PART 4: SELF-LIMITING LEVELS OF USE

There is no technological or organoleptic limitation to the concentration of the blend of the three strains *Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, or *Bifidobacterium bifidum* Rosell®-71, or of any of these three notified strains individually, which may be added to infant formula.

PART 5: EXPERIENCE BASED ON COMMON USE IN FOOD

The conclusion that the intended use of the blend of the three strains *Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, and *Bifidobacterium bifidum* Rosell®-71, or of any of these three notified strains individually, is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958.

PART 6: NARRATIVE

Sections:

6.1. Recognized Safety of Lactic Acid Bacteria

The bacterial biota along the entire intestinal tract is extremely complex and includes an estimated 10^{13} - 10^{14} or more bacteria representing over 400 different species (Zetterstrom et al. 1994; Edwards and Parrett 2002) or more than 2000 phylotypes (McFall-Ngai 2006). These indigenous bacteria break down some food components into more easily assimilable forms (Edwards and Parrett 2002), support local immune

responses (Zetterstrom et al. 1994), and contribute to an environment that resists colonization by potential pathogens (Heavey and Rowland 1999). Probiotic strains are selected to impart beneficial effects on the host and on the composition and or metabolism of the intestinal microbiota without causing adverse changes (e.g., invasion of the epithelial cells, degradation of the intestinal mucin layer, production of toxins, transference of antibiotic resistance) that would imperil the health or nutritional status of the host.

Lactobacilli have been consumed on a daily basis since humans started using fermented milks as food, including the probiotic use of certain *Lactobacillus* species for more than 75 years (Salminen et al. 1998), and indeed were almost certainly widely consumed even before that time since they are normal inhabitants of green plant material. Bernardeau et al. (2006) noted that, "*lactobacilli* are ubiquitous, being found wherever substances rich in carbohydrates are available." These authors reported that in healthy humans, "*lactobacilli* are normally present in the oral cavity (10^3- 10^7 cfu/g), the ileum (10^3-10^7 cfu/g), and the colon (10^4- 10^8 cfu/g) and they are the dominant microorganism in the vagina."

A Food and Agriculture Organization and World Health Organization expert consultation (FAO/WHO 2001) noted that, "*lactobacilli* have a long history of use as probiotics without established risk to humans, and this remains the best proof of their safety'' and concluded that, "no pathogenic or virulence properties have been found for *lactobacilli*."

Discussing the use of probiotics in primary care pediatrics, Cabana et al. (2006) observed that the optimal dose of probiotics remains an area of active investigation, but noted that, "Although no specific pediatric dose has been established in general, there are no known reports of 'toxicity' associated with exceeding a specific dose in either adults or children."

Vandenplas et al. (2007) observed that *lactobacilli* and other probiotics "do not colonize the gastro-intestinal tract as they become undetectable a few days after stopping the administration. This results in the absence of any risk for long-term side effects". One study (Schultz et al. 2004), found that infants born to mothers who had received daily oral doses of $2xl0^9$ cfu *L. rhamnosus* strain GG (LGG) during the 30-36 weeks of their pregnancies had detectible LGG strains in their feces for extended periods, with strain identification confirmed by molecular methods. All of the 4 infants delivered vaginally and 1 of 2 infants delivered by Caesarian section were shedding LGG at 1 and 6 months of age. Three children still had detectible fecal LGG at 12 months and 2 at 24 months; none had detectible LGG in their feces at 36 months of age; none of the mothers, on the other hand, exhibited evidence of LGG colonization by 1 month after delivery.

In an article addressing the safety of *lactobacilli* and *bifidobacteria*, Borriello et al. (2003) suggested that "classical" approaches to evaluating safety are not appropriate for these commensal bacteria:

"*Lactobacilli* and *bifidobacteria* are ubiquitous in the diet and in the healthy large intestine soon after birth. A classical risk assessment approach, similar to that used for pathogens, is not possible or warranted. Some studies of *lactobacilli* have attempted to define virulence factors. Such classical approaches, although useful for known pathogens, are inherently flawed when applied to normal commensals, *lactobacilli,* or bifidobacteria. In the case of the risk assessment approach for pathogens, pathogenicity is demonstrated and is normally a consequence of several properties, including colonization factors and virulence factors, acting in concert. Frequently, such factors as adhesion are considered to be virulence factors when pathogens are studied. However, mucosal adhesion and other colonization factors are essential features of most commensals. For example, there is a distinct mucosal-associated flora in the gastrointestinal tract. There is little value in screening organisms of low clinical significance and with no proven virulence determinants for such characteristics as potential virulence factors, particularly in the absence of gastrointestinal commensals as comparative controls".

Borriello et al. (2003) argued that the risk of bacteremia from probiotic lactobacilli and bifidobacteria is well under 1 in a million and concluded that, based on the overall risk from this or other adverse endpoints, "consumption of such products presents a negligible risk to consumers, including immunocompromised hosts."

In a similar vein, Bernardeau et al. (2008) suggested that, "The bibliographical data support the hypothesis that the ingestion of *Lactobacillus* is not at all hazardous since lactobacillemia induced by food, particularly fermented dairy products, is extremely rare and only occurs in predisposed patients."

6.2. History of Consumption of Notified Bacterial Strains

6.2.1. History of Consumption of *Lactobacillus helveticus Rosell®-52 (R0052)*

As previously noted, this strain of *Lactobacillus helveticus* was isolated from a dairy culture. It is a proprietary culture provided to Lallemand Health Solutions (formerly known as Institut Rosell) in 1990 by the company, Weinstein Nutritional Products of California, USA. There is no documented record of consumption of the strain, but it is possible that it has been consumed worldwide, including by Americans. There are no records of any reported adverse events associated with consumption of dairy products containing the strain.

Lactobacillus helveticus R0052 has been sold worldwide for many years as a powder or as a part of the following blends:

- Probiokid®, a combination of *Lactobacillus helveticus* Rosell®-52 (R0052) (80%), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) (10%) and *Bifidobacterium bifidum* Rosell®-71 (R0071) (10%). Probiokid® is commercialized as a blend of the above named strains ($3x10^9$ cfu/sachet, corresponding to $2.4x10^9$ cfu of *Lactobacillus helveticus* Rosell®-52 [R0052]). Probiokid® is used in infants, toddlers, and children, with a recommended dose of $3x10^9$ to $5x10^9$ cfu per day. Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime. Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, United Kingdom, etc…
- Probio'Stick®, a combination of *Lactobacillus helveticus* Rosell®-52 (R0052) (89.4%) and *Bifidobacterium longum* ssp. *longum* R0175 (10.6%). Each sachet of Probio'Stick® contains $3x10^9$ cfu, corresponding to $2.7x10^9$ cfu of *Lactobacillus helveticus* Rosell®-52 (R0052) per dose, with recommended one dose per day. The target population using Probio'Stick® is adults. Probio'Stick® was first marketed in 2006 in Canada and Poland and has since been sold in other countries in the worldwide.
- Lacidofil®, a combination of *Lactobacillus rhamnosus* R0011 (95%) and *Lactobacillus helveticus* Rosell®-52 (R0052) (5%). Each capsule of Lacidofil® contains $2x10^9$ cfu, corresponding to $0.1x10^9$ cfu of *Lactobacillus helveticus* Rosell®-52 (R0052) per dose. Lacidofil® has recently been the subject of a review (Foster et al. 2011). The target population of Lacidofil® is infants, children, and adults, corresponding to *L. helveticus* R0052 consumption between 0.1 and 0.6 billion cfu per day. Lacidofil® is consumed from the age of 1 month in France and in Poland under the statute "Food for Special Medical Device." No adverse effect has been declared by the event adverse reporting system of these authorities. The first marketing authorization for Lacidofil® was obtained in Ukraine in 1995, and the product has since been sold in other countries in the worldwide.
- Protecflor®, a combination of *Bifidobacterium longum* ssp. *longum* R0175 (33%), *Lactobacillus helveticus* Rosell®-52 (R0052) (33%), *Lactobacillus rhamnosus* R0011 (33%) and *Saccharomyces boulardii* (125 mg). Each capsule of Protecflor® contains $5x10^9$ cfu of bacteria, corresponding to $1.67x10^9$ cfu of *L. helveticus* Rosell®-52 (R0052) per dose and per day. The first launch of Protecflor® was in 2007 in Australia, France, India, Serbia and Montenegro, UK,

and has since been sold in other countries in the worldwide. The target population is adults. No adverse effect has been declared by the authorities.

- Oralis SB® powder® a combination of *Lactobacillus helveticus* Rosell®-52 (R0052) (54 %), *Lactobacillus rhamnosus* R0011 (29%), *Bifidobacterium longum* ssp. *longum* R0175 (10%) and *Saccharomyces cerevisiae* var *boulardii (7%).* Each sachet of Oralis SB® powder contains $1.25x10^9$ cfu of bacteria, corresponding to $0.67x10^9$ cfu of *L, helveticus* Rosell®-52 (R0052) per dose and between 0.67 and $1.34x10^9$ cfu per day. The first launch of Oralis SB® powder® was in India under the Brand name Darolac® by the company Aristo Pharmaceutical and is approved in Canada as a Natural Health. The target population is children above 7 years old and adults.

Additionally, *Lactobacillus helveticus* Rosell®-52 (R0052) has also been extensively and widely marketed by Lallemand Health Solutions as a combination (with other strains) in more than 170 other formulas and this in more than 20 different countries (USA, Canada, UK, France, Italy, Spain, Sweden, Poland, India, China, Australia, South Africa, Mexico, Malaysia, Indonesia, etc.). Moreover, this strain has also been purchased in more than 10 countries by subcontractors who use it in various probiotic formulas.

Moreover *Lactobacillus helveticus* Rosell®-52 (R0052) appears on a peer-reviewed list of strains that have been recognized for their probiotic properties in different scientific articles (Mercenier et al. 2002, Johnson-Henry et al. 2004).

As presented above, *Lactobacillus helveticus* Rosell®-52 (R0052) has been sold worldwide for many years, and no countries with a nutrivigilance system (e.g. France, Canada, etc.) have reported adverse events after the consumption of this strain in adults and/or children. This shows that the intake of *Lactobacillus helveticus* Rosell®-52 (R0052) is safe at $3x10^9$ cfu per day in infants and adults.

6.2.2. History of Consumption of *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033) has been sold worldwide for many years as a powder or as a part of Probiokid®, a blend providing $3x10^9$ cfu/sachet, corresponding to $3x10^8$ cfu of *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033). Probiokid® is used in infants, toddlers, and children. Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime. Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, United Kingdom, etc...

Additionally, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) has also been extensively marketed by Lallemand Health Solutions as a combination (with other strains) in more than 40 other formulas with no reports of adverse effects.

6.2.3. History of Consumption of *Bifidobacterium bifidum* Rosell®-71 (R0071)

Bifidobacterium bifidum Rosell®-71 (R0071) has been sold worldwide for many years as a powder or as a part of Probiokid®, a blend providing $3x10^9$ cfu/sachet, corresponding to $3x10^8$ cfu of *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033). Probiokid® is used in infants, young children and children. Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime. Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, United Kingdom, etc…

Additionally, *Bifidobacterium bifidum* Rosell®-71 (R0071) has also been extensively marketed by Lallemand Health Solutions as a combination (with other strains) in more than 80other formulas with no reports of adverse effects.

6.2.4. History of Consumption of Probiokid®, the Combination of Three Bacterial Strains

The blend of *Lactobacillus helveticus* Rosell®-52 (R0052*)* (80)%, *Bifidobacterium longum* ssp. *Infantis* Rosell®-33 (R0033) (10%) and *Bifidobacterium bifidum* Rosell®-71 (R0071) (10%) is consumed as part of the product Probiokid® at the same ratio 80:10:10, used in infants, young children and children. Probiokid® was first launched as a health food in China and its marketing started in October 2002, under the trade name Biostime. Since 2002, more than 1.2 billion of Probiokid® sachets have been sold in several countries.

Probiokid® is included in an ongoing program of pharmacovigilance for monitoring adverse events. Probiokid® has also been the subject of a Periodic Safety Update Report (PSUR) which outlines the safety profile of this product. The first PSUR covers the period from 01 January 2006 through 31 December 2012. During that time, a total of 574,205,358 sachets of Probiokid® were sold globally and 4 non-serious adverse events were reported. For the second PSUR, covering the period from 01 January 2013 through 31 December 2016, a total of 564,810,364 sachets of Probiokid® were sold. No non-serious or serious adverse events were reported.

Additionally, there were no actions for safety reasons initiated by any health authority concerning Probiokid® sachets. A regular review of the published scientific literature detected no reports of adverse events related to the intake of Probiokid® or any of the strains of which it is composed, *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), and *Bifidobacterium bifidum* Rosell®-71 (R0071).

The global evaluation of the safety of Probiokid® sachets during the reporting period did not reveal significant information to be notified on the safety profile of the product.

6.3. Safety Parameters

6.3.1. Ability to Adhere to Intestinal Cells

The ability to adhere to mucosal surfaces is an interesting property for a probiotic. It confers a competitive advantage important for bacterial maintenance and colonization in the human gastrointestinal tract.

Adhesion to gastric epithelial cells has often been suggested as selection criterion for probiotic potency (FAO/WHO 2002). However, there is no scientific evidence to support such a claim. While adhesion may be necessary for some effects such as direct competition for epithelial cell binding sites with certain adherent forms of pathogenic microbes such as enteropathic and enterohemorrhagic *E. coli* or *H. pylori* (Johnson-Henry et al. 2004), there is no evidence that adhesion is required for other pharmacodynamic properties of a strain as, for example, immune modulation and pathogen inhibition by secreted substances (e.g., lactic acid, hydrogen peroxide, bacteriocins).

Although adherence of probiotic bacteria to intestinal surfaces is not confirmed to be required for health benefits, it has been hypothesized to be involved in establishing residence, for stimulation of the immune system, and for antagonistic activity against enteropathogens (Gopal et al. 2001). Nevertheless, some concern has been expressed that high adhesion capability, a characteristic of pathogens, may facilitate platelet aggregation and bacterial infectivity (Kirjavainen et al. 1999). *In vitro* assays of the adherence ability of bacterial strains are commonly conducted; however, their ability to predict *in vivo* adherence is uncertain. In an *in vitro* evaluation of 8 bacteremia-associated *Lactobacillus* strains, Kirjavainen et al. (1999) found no relationship between adherence to Caco-2 cells, ileostomy glycoproteins, or human intestinal mucosa and either platelet aggregation or infectivity.

To date, the available information on the adhesion properties of *Bifidobacteria* is still limited (He et al. 2001). Scientists have developed *in vitro* adherence tests with human cells grown in tissue cultures to measure this adherence.

- **Lactobacillus helveticus Rosell®-52 (R0052)**

The capacity of *Lactobacillus helveticus* R0052 to bind to epithelial cells has been demonstrated. It shows strong adherence to the surface of different cell types (Wallace et al. 2003; Kostrynska 2004a [unpublished] 2004b [unpublished]; Shin and Wallace 2005 [unpublished]), particularly to HT-29 cells and to the squamous cell line KYSE-30. The scanning electron micrograph below shows R0052 adhering to the surface of intestinal epithelial cells.

Table 23. Adherence of *L. helveticus R0052* to Different Cell Types (Demonstrated *in Vitro*).

Cell type	Adherence
T84 human intestinal epithelial	Yes
HT-29 human intestinal epithelial	Yes
KYSE-30 esophageal squamous	Yes
HEp-2 laryngeal epithelial	Yes

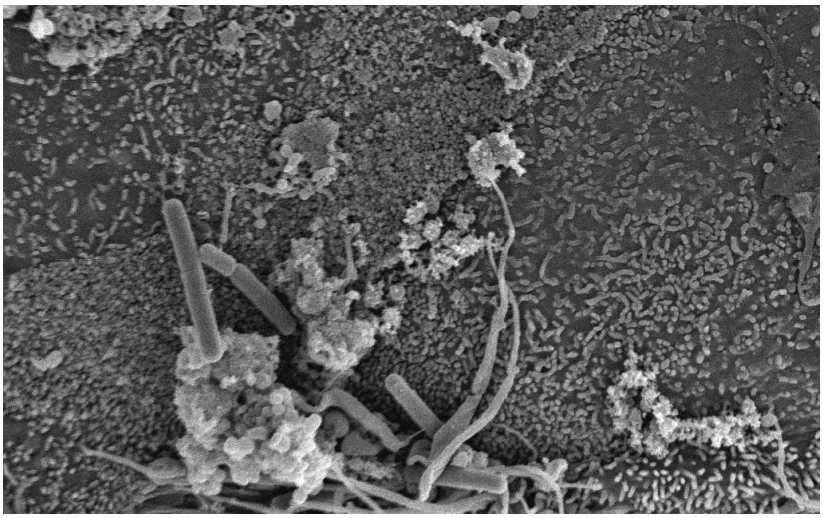


Figure 18. Scanning Electron Micrograph Image Showing *L. helveticus R0052* Adherence to T84 Intestinal Epithelial Cells in Culture (Magnification X4300). (Scanning Electron Micrograph photo courtesy of Dr. P. Sherman, Research Institute, The Hospital for Sick Children, Toronto, Ontario.)

Adhesive Capacity of R0052 to HT-29 Intestinal Epithelial Cells

L. helveticus R0052 was added to individual wells of HT-29 cells in triplicate at a concentration of $1.0x10^6$ cfu/mL and incubated at 37ºC, 5% CO_2 for 3 hours (Shin and Wallace 2005; unpublished). Following incubation, cells were rinsed gently with PBS to remove unbound bacteria and treated with 1mL of 0.05% Trypsin-EDTA for 30 minutes at 37ºC, 5% CO_2 to detach HT-29 cells/adhesive bacteria. Following centrifugation, spent supernatant was removed and HT-29 cells were lysed by the addition of 100µL of 0.1% bovine albumin. The resulting solution was serially diluted and standard plate counts were performed on MRS agar at 37ºC for 48H. Control wells containing HT-29 cells alone were treated in a similar manner. Cell counts were performed using a hemocytometer prior to the addition of bovine albumin, allowing for quantitative determination of the binding capacity of the bacterial strain. As illustrated in Figure 19, the different assays demonstrate an average number of 1.71 R0052 adherent cell per HT-29 cell.

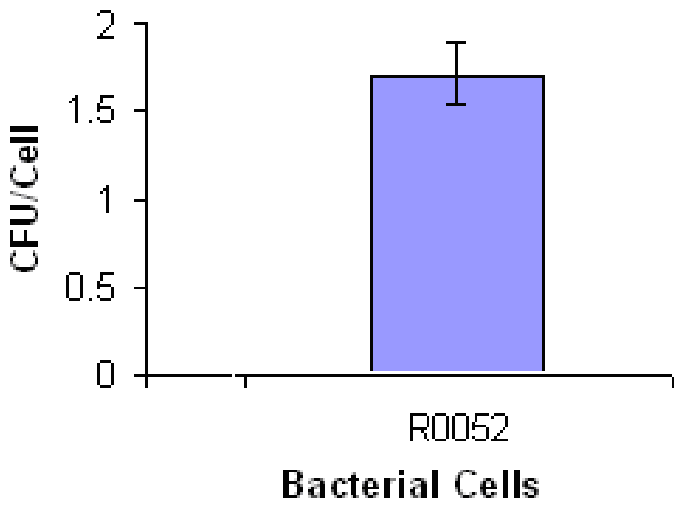


Figure 19. Binding Capacity of *L. helveticus* R0052 to HT-29 Epithelial Cells *in Vitro.*

Direct Counts of *Lactobacillus* Adherence to KYSE-30 Cells

KYSE-30 cells were seeded into 12 well plates containing sterile circular glass coverslips and grown until confluent at 37°C, 5% CO_2 in 2 ml of appropriate complete growth media (Kostrynska 2004a; unpublished). *Lactobacillus helveticus R0052* was grown in MRS media for 24 hours at 37°C under anaerobic conditions. After the incubation, bacterial cultures and epithelial cells were washed (with Phosphate Buffered Saline and Hank's Balanced Salt Solution respectively) and then 100µl of the bacterial suspension in 1.9 ml of complete growth medium was added to the appropriate well in duplicate and incubated for 1h at 37°C, 5% CO_2. Each well was then washed and fixed with methanol for 5 minutes. Coverslips were air dried and Gram strained. The total number of bacteria and epithelial cells in 10 fields were counted under 1000x magnification and the average number of bacteria per 100 epithelial cells was determined. Each experiment was performed in triplicate (Figure 20).

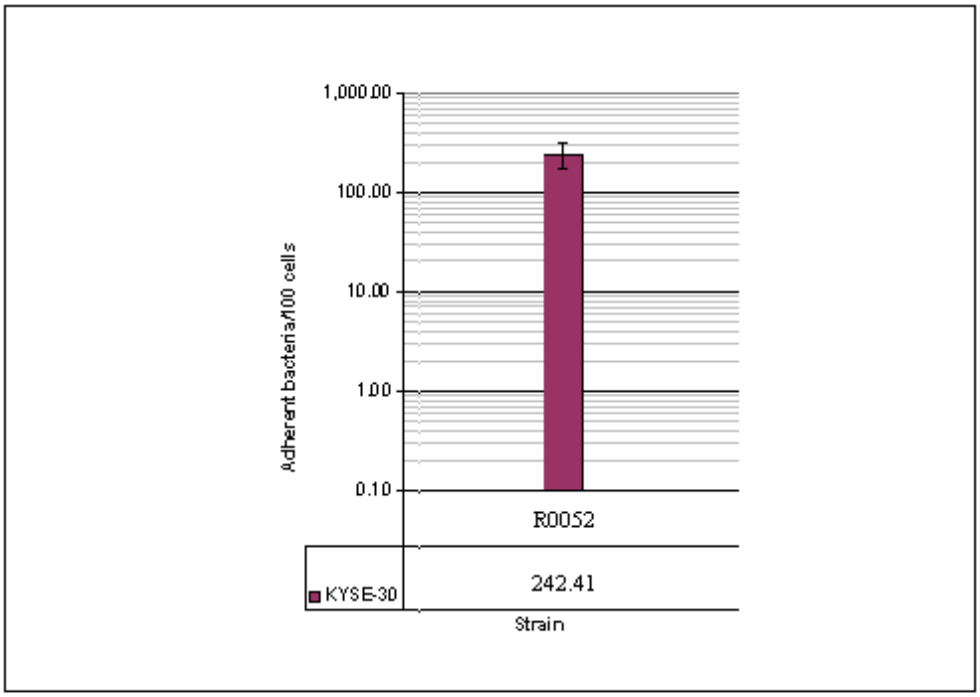


Figure 20. Adherence of *L. helveticus* R0052 to KYSE-30 Epithelial Cells *in Vitro* (Error Bars Indicate Standard Deviation).

Binding Curve of Radiolabeled *L. helveticus* R0052 to KYSE-30 Monolayer

KYSE-30 cells were seeded and grown until confluent at 37°C, 5% CO_2 (Kostrynska 2004a; unpublished). *L. helveticus* was grown in MRS media for 24h at 37°C under anaerobiosis. Following this incubation period, the R0052 culture was subbed 1/10000 in fresh MRS containing 100μCi/ml and grown for additional 18h. Radiolabeled R0052 bacteria were washed and resuspended to 10^9 cfu/ml. Scintillations were counted and cfu/CPM values were determined. KYSE-cells were washed and radiolabeled bacteria were serially diluted and added to KYSE-30 wells in triplicate and incubated at 37°C for 1h. Scintillations were counted for 2 minutes and cfu values were extrapolated. Approximately 3.4% of R0052 added bound to the KYSE-30 monolayer before saturation was reached (Figure 21).

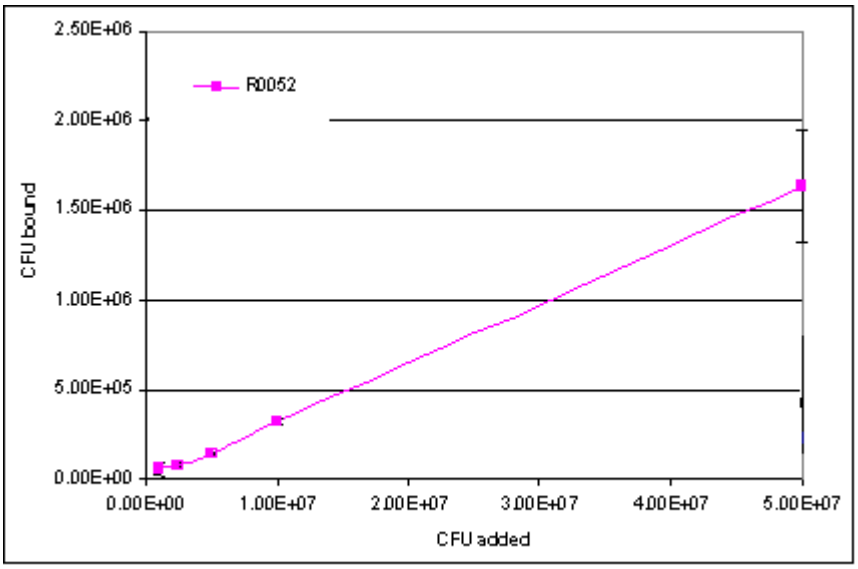


Figure 21. Binding Curve of *L. helveticus* R0052.

- ***Bifidobacterium longum ssp. infantis Rosell®-33 (R0033)***

The capacity of *Bifidobacterium longum* ssp. *infantis* R0033 to bind to epithelial cells has been demonstrated. It showed binding ability to surface of HT-29 intestinal epithelial cells (internal data) and Caco-2 intestinal epithelial cells (Kostrzynska Dixon and Lepp 2002; Kostrzynska, Dixon and Lepp 2002a) in *in vitro* conditions. The adhesion of R0033 was 4.68±2.51% when the HT-29 was grown 48 hours and was 5.01±3.33% when HT-29 reached full confluence after 15 days (Figure 22).

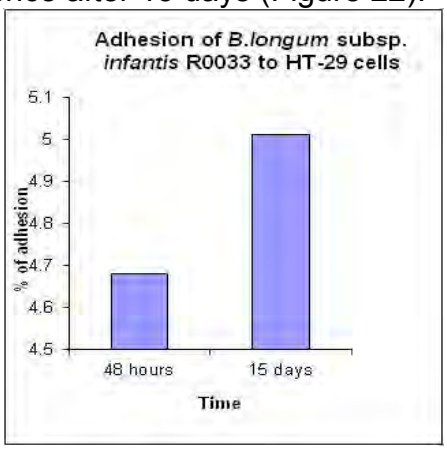


Figure 22. Adherence of *B. longum* ssp. *infantis* R0033 to HT-29 Intestinal Epithelial Cells (*in Vitro*).

Scanning Electron Microscopy of *B. longum* ssp. *infantis* R0033 Adhering to Caco-2 Intestinal Cells (Kostrzynska, Dixon and Lepp 2002a).

Caco-2 cells were grown in Petri plates on glass slides until ~80% confluent and then incubated with *B. longum* ssp. *infantis* R0033 (~5x10^9 cfu/ml) in MEM media for 1h at 37C in 5% CO_2. They were then washed 5 times with phosphate buffered saline (PBS:pH 7.6) and fixed with glutaraldehyde for 50 minutes followed by osmium tetrachloride for 15 minutes. Following dehydration through an ethanol series, the slides were critical point dried, sputter coated and viewed under a scanning electron microscope.

A. Light microscope image of *B. longum* ssp. *infantis* R0033 adhering to Caco-2 cells. B-D. Scanning electron microscope (SEM) images of *B. longum* ssp. *infantis* R0033. C. Intimate interaction between R0033 and microvilli D. insert of C (Figure 23).

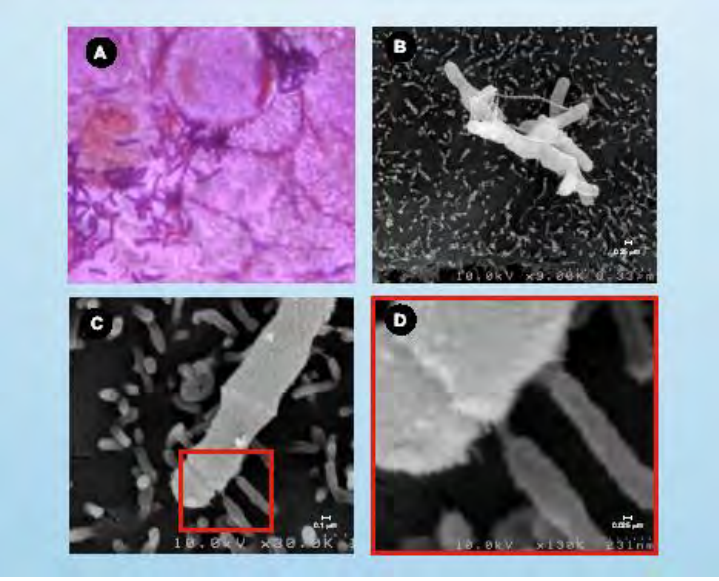


Figure 23. Adherence of *B. longum* ssp. *infantis* R0033 to Caco-2 Intestinal Epithelial cells (*in Vitro*).

Glycosphingolipid Binding of *B. longum* ssp. *infantis* R0033 (Kostrzynska, Dixon and Lepp 2002)

A) High performance thin-layer chromatography (HPTLC) of various glycolipids developed in chloroform:methanol:water (60:35:8) stained with anisaldehyde reagent. B) Duplicate chromatogram overlaid with biotinylated R0033 bacteria. Abbreviations are: **GluCer**: Glucocerebrocides; **GalCer**: Galactocerebrosides; **LacCer**: Lactocerebrocides;

Glo: Globoside; **GM1**: Monosialoganglioside-Gm1; **Ga1:** Gangliotetraosylceramide; **Sulf**: Sulfatides. *B. longum* ssp. *infantis* bound to the acidic glycosphingolipid sulfatide (**Sulf**). In addition, selective binding of *B. longum* ssp. *infantis* to gangliotetraosylceramide (Ga1) was detected (Figure 24).

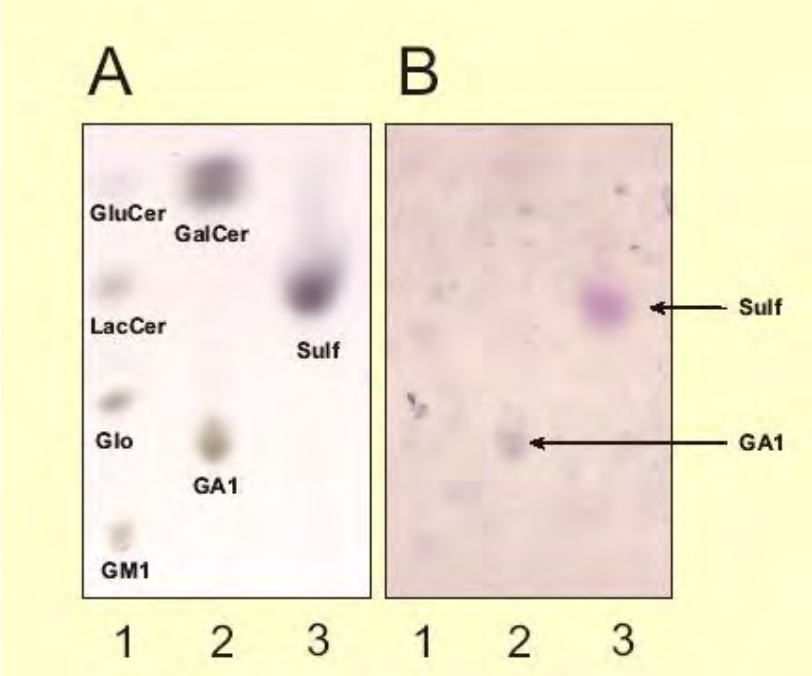


Figure 24. Binding of *B. longum* ssp. *infantis* R0033 to Glycolipids.

Binding to Immobilized Carbohydrates (Kostrzynska, Dixon and Lepp 2002). Biotinylated R0033 cells were overlaid onto nitrocellulose membrane spotted with either A) BSA B) BSA-conjugated 2` fucosyllactose, or C) BSA-conjugated Lewis b tetrasaccharide. The carbohydrate structures are as follows: B) α-Fuc[1→2]-β-Gal[1→4]-Glc and C) α-Fuc[1→2]-β-Gal[1→4] - [α-Fuc (1→4)]- GlcNac-Lac spacer.

Binding to both 2` fucosyllactose and Lewis b tetrasaccharide was detected (Figure 25).

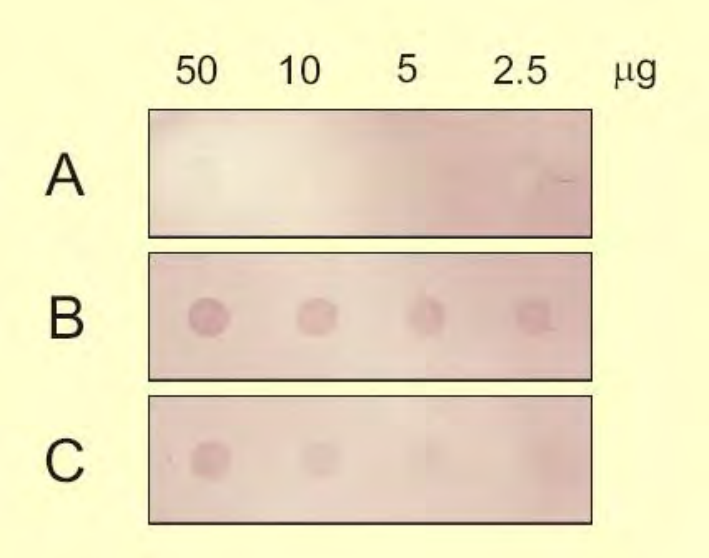


Figure 25. Binding of *B. longum* ssp. *infantis* R0033 to Fucosylated Sugars.

Inhibitory Effect of Carbohydrates and Glycolipids in the Binding of R0033 to Caco-2 Cells (Kostrzynska, Dixon and Lepp 2002)

B. longum ssp. *infantis* R0033 grown on reinforced clostridial agar was suspended in PBS and incubated with the appropriate carbohydrate or glycolipid at 37°C for 1h. Control bacteria were incubated with PBS alone. The suspension was then added to a well containing a monolayer of Caco-2 cells and incubated at 37° for 1 h. The monolayer was then washed, fixed in methanol and Gram stained. The average number of bacteria adhering per 100 Caco-2 cells was determined microscopically and expressed relative to controls. Abbreviations are **GlcN**: glucosamine; **2`FL :** 2` fucosyollactose; **3` FL**: 3`-fucosyllactose; **Leb**: Lewis b tetrasaccharide; **Sulf :** sulfatides and **GA1:** gangliotetraosylceramide.

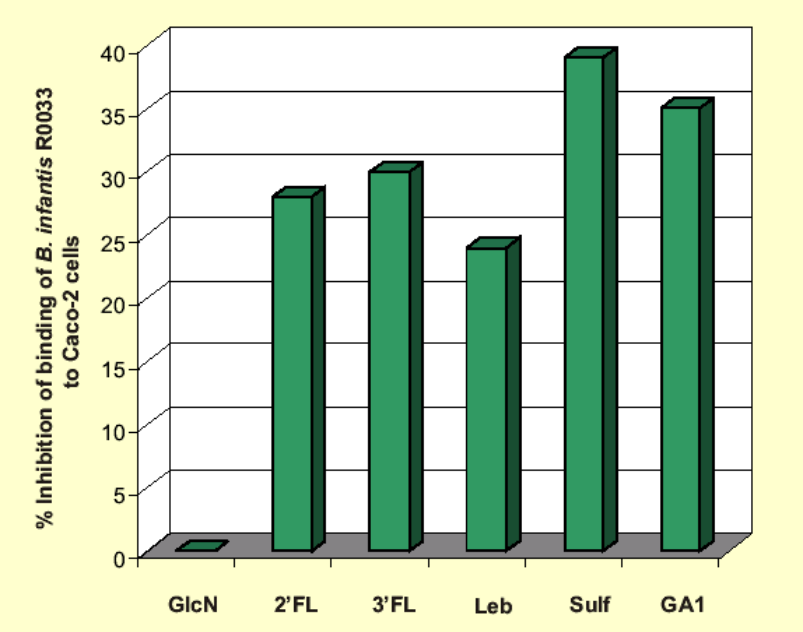


Figure 26. Binding Specificity of *B. longum* ssp. *infantis* R0033 to Caco-2 Intestinal Epithelial Cells.

Fucosylated sugars, including 2`-fucosyllactose and the Lewis b blood group determinant were repeatedly found to inhibit adherence. Sulfatide and GA1 also inhibited binding of this strain to Caco-2 cells, indicating that these glycolipids may serve as adhesion receptors for R0033. Fucosylated compounds, sulfatide and GA1 have all been demonstrated to act as receptors for *Helicobacter pylori* (Ilver et al. 1998; Mukai et al. 2002). GA1 has also been proposed to act as a receptor for a number of other pathogens, including enterotoxigenic *E. coli* (ETEC) (Fujiwara et al. 1997; Oro et al. 1990). Therefore, it appears that *B. longum* ssp. *infantis* R0033 shares common binding specificities with several pathogenic microorganisms.

- ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

The capacity of *Bifidobacterium bifidum* Rosell®-71 (R0071) to bind to epithelial cells has been demonstrated *in vitro*. R0071 adheres to the surface of HT-29 intestinal epithelial cells (internal data). The % adhesion of R0071 to HT-29 intestinal epithelial cells was 6.90±3.00 when co-incubated 3 hours with HT-29 grown 48 hours.

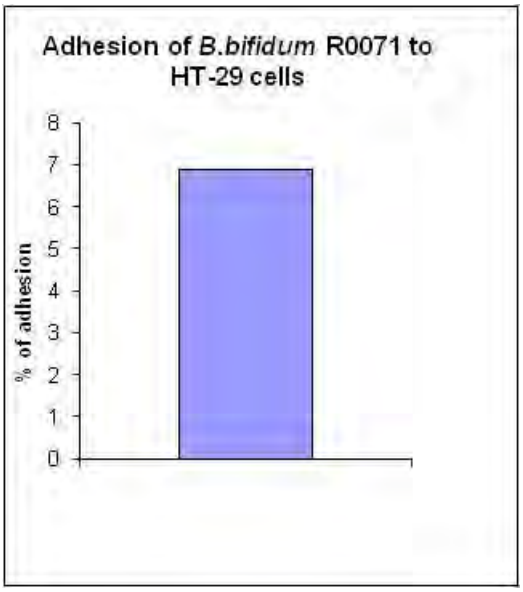


Figure 27: Adherence of *B. bifidum* R0071 to HT-29 Intestinal Epithelial Cells (*in Vitro*).

6.3.2. Infectivity

Cases of infection by lactic acid bacteria are extremely rare. Reid and Hammond (2005) asserted that, “The safety record of probiotics is remarkable considering that more than 20 billion doses are estimated to be used each year”. Over the past 30 years there have been about 180 published cases of bacteremia and 69 cases of endocarditis putatively caused by lactobacilli (Aguirre and Collins 1993; Gasser 1994; Donohue et al. 1992). The majority of these cases have occurred in patients with compromised immune status or mucosal barrier function due to underlying conditions such as heart disease or diabetes or therapeutic treatment (e.g., dental surgery). Boyle et al. (2006) stated firmly,

"All cases of probiotic bacteremia or fungemia have occurred in patients with underlying immune compromise, chronic disease, or debilitation, and no reports have described sepsis related to probiotic use in otherwise healthy persons".

Lactobacillus helveticus, *Bifidobacterium infantis*, and *Bifidobacterium bifidum* are organisms recognized for their long history of safe use. They are included in an inventory, assembled by the International Dairy Federation in collaboration with the European Food and Feed Cultures Association, of microorganisms that have a documented history of safe use in food (Bourdichon et al. 2012).

The *Lactobacillus, Bifidobacterium infantis, and Bifidobacterium bifidum* taxonomic groups are not known to contain toxin producers or strains that possess virulence factors (Gasser 1994). Therefore, their pathogenic potential is extremely low. Only a limited number of adverse reactions have been published and, overall, consideration should be given to the condition of the consumer or patient. In fact, infection cases reported invariably concern individuals in a fragile state with underlying conditions (Salminen et al. 1998).

6.3.3. Undesirable Metabolic Activity

6.3.3.1 D-Lactate Production

All probiotic and intestinal lactic acid bacteria produce some amount of D-lactate (from 1% of all lactate produced up to 97%, depending on the strain; with 40% being a typical amount). Intestinal bacteria express either or both D(-) or L(+) lactate specific dehydrogenase (Hove and Mortonsen 1995; Kochhar et al. 1992). Carbohydrates such as hexoses (glucose, galactose, fructose) ingested into the intestinal tract are fermented by bacterial glycolytic pathways to pyruvate and either L(+)- or D(-)-lactate. Additionally, some *Lactobacillus* strains have DL-lactate racemase which catalyzes the conversion between D(-) and L(+)- lactate (Hove and Mortonsen 1995). Thus, colonic D(-)-lactate may be formed from pyruvate by bacterial D(-)-lactate dehydrogenase or from L(+)-lactate by racemization (Hove 1998). L(+)- and D(-)-lactate are intermediary products that other colonic bacteria can metabolize to short chain fatty acids [i.e. acetate, butyrate, propionate] and used for energy by mucosal cells of the colon (Hove and Mortonsen 1995).

The only medical indication that D(-)-lactate producing strains should not be used is derived from older studies in which infants were fed formulas that were acidified with known amounts of D(-) and L (+)-lactate (Stolley and Droese 1971). Subsequent studies with acidified formulas have not supported these initial findings. The acidification was a direct result of the addition of chemical lactic acid and not naturally occurring acidification due to the fermentation of food matter.

Connolly and Lönnerdal (2004) wrote an interesting review on D(-)-lactic acid producing bacteria. After reviewing what D-lactic acid is and its metabolism, they reported whether orally administered D(-) lactic acid is toxic in human adults and newborn infants or not. Here are their conclusions, based on the available evidence:

1. There is no evidence to show that the normal gastrointestinal tract biota can induce D(-)-lactic acidosis in the healthy human adult or infant.
2. D(-)-lactic acid acidosis only occurs in subjects with a disturbed gastrointestinal function following bowel resection.
3. Well-controlled clinical trials (doses of 10^5 to 10^9 cfu/day for 28-30 days) where the DL-lactic acid producing probiotic bacteria *Lactobacillus reuteri* was given to over 160 human newborn term and preterm infants clearly indicated that clinical signs of acidosis did not occur after *L. reuteri* administration at any dose tested.
4. Exposure of infants to the probiotic bacterium *L. reuteri* does not result in abnormal levels of D(-)-lactic acid in the blood.
5. There is no valid reason to exclude the supplementation of indigenous human *Lactobacillus spp*. to the newborn human infant on the basis of the stereo-isomers of lactic acid these bacteria produce.

- ***Lactobacillus helveticus* Rosell®-52 (R0052)**

Lactobacillus helveticus Rosell®-52 (R0052) produces L(+)-lactate (6.19 g/L) and D(-)-lactate (9.08 g/L), with the latter representing about 59% of all lactate produced. The UV test kit for the determination of D-/L-lactic acid from Xygen Diagnostics Inc was used for the quantification. The strain was grown in MRS broth for 16-18 hours at 37°C.

- ***Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)**

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033) produces only L(+)-lactate (2.76 g/L). The D-lactate isomer is not produced. The UV test kit for the determination of D-/L-lactic acid from Xygen Diagnostics Inc was used for the quantification. The strain was grown anaerobically in M30 broth for 16-18 hours at 37°C.

- ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

Bifidobacterium bifidum Rosell®-71 (R0071) produces only L(+)-lactate (3.7 g/L). The D-lactate isomer is not produced. The UV test kit for the determination of D-/L-lactic acid from Xygen Diagnostics Inc was used for the quantification. The strain was grown anaerobically in M30 broth for 16-18 hours at 37°C.

6.3.3.2. Bile Salt Deconjugase Activity

Bile salts are steroids with detergent properties which are used to emulsify lipids in foodstuff passing through the intestine to enable fat digestion and absorption through

the intestinal wall. They are secreted from the liver, stored in the gall bladder, and passed through the bile duct into the intestine when food is passing through. Biosynthesis represents the major metabolic fate of cholesterol, accounting for more than half of the 800mg/day of cholesterol that the average adult uses up in metabolic processes. By comparison, steroid hormone biosynthesis consumes only about 50 mg of cholesterol per day. Much more than 400 mg of bile salts is required and secreted into the intestine per day, and this is achieved by re-cycling the bile salts.

Most of the bile salts secreted into the upper region of the small intestine are absorbed along with the dietary lipids that they emulsified at the lower end of the small intestine. They are separated from the dietary lipid and returned to the liver for re-use. The most abundant of the bile salts in humans are cholate and deoxycholate, and they are normally conjugated with either glycine or taurine to give glycocholate or taurocholate respectively. The conjugation is important in identifying the bile salt for re-cycling back to the liver. When these bile salts are deconjugated, that is, glycine or taurine is removed, then the resulting free bile salt will form a precipitate and will not be reabsorbed. The precipitate of bile salt will be excreted with the feces. By increasing the amounts of bile salt excreted, the level of circulating cholesterol can be reduced. The deconjugation of bile salts is achieved through the activity of bile salt hydrolases (BSH) which are produced by intestinal bacteria. *Enterococci* and *Clostridia* contain some of the highest levels of bile salt deconjugase activity (Knarreborg et al. 2002) but BSH activity is also found in many *Bifidobacteria* and some *Lactobacilli*. It should be noted here that the ability of a microbe to resist bile salt inhibition does not appear to be due to its capacity to hydrolyze bile salts (Moser and Savage 2001).

It has been suggested that certain potential probiotic microbes with bile salt hydrolase activity could be important for maintaining hypocholesterolemia and may be prophylactic for arteriosclerosis (Rhee et al. 2002).

- ***Lactobacillus helveticus* Rosell®-52 (R0052)**

An internal study was performed to determine the presence of bile salt deconjugase in various Rosell LAB strains. It was shown that *L. helveticus* Rosell®-52 (R0052) does not possess any bile salt deconjugase activity (Belvis and Wallace 2004; unpublished). It was shown that R0052 possesses two partial bile hydrolase genes but they are not active. It does not cause bile acid-induced diarrhea.

Table 24. Detection of Bile Salt Deconjugase Activity for *L. helveticus* Rosell®-52 (R0052).

Strain	Growth medium	Incubation conditions	Control	Interpretation	Bile salt deconjugase activity
L. helveticus R0052	A MRS Agar plate supplemented with 0.5% (w/v) taurodeoxycholic acid (TDCA)	Plates incubated for 5 days at 37°C under anaerobic conditions	Unsupplemented MRS agar plate	Bile salt deconjugase activity is manifest by the presence of clear precipitate halos around isolated colonies or opaque , granular white colonies compared to control colonies grown on unsup-plemented agar	Negative

- ***Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)**

An internal study was performed to determine the presence of bile salt deconjugase in various Rosell LAB strains. It was shown that *B. longum* ssp. *infantis* R0033 exhibits bile salt deconjugase activity (Belvis and Wallace 2004; unpublished).

Table 25. Detection of Bile Salt Deconjugase Activity for *B. longum* ssp. *infantis* R0033.

Strain	Growth medium	Incubation conditions	Control	Interpretation	Bile salt deconjugase activity
B. longum ssp. *infantis* R0033	A RCM Agar plate supplemented with 0.5% (w/v) taurodeoxycholic acid (TDCA)	Plates incubated for 5 days at 37°C under anaerobic conditions	Unsupplemented RCM agar plate	Bile salt deconjugase activity is manifest by the presence of clear precipitate halos around isolated colonies or opaque , granular white colonies compared to control colonies grown on unsup-plemented agar	Positive

- ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

An internal study was performed to determine the presence of bile salt deconjugase in various Rosell LAB strains. It was shown that *B. bifidum* Rosell®-71 (R0071) exhibits bile salt deconjugase activity (Belvis and Wallace 2004; unpublished).

Table 26. Detection of Bile Salt Deconjugase Activity for *B. bifidum* Rosell®-71 (R0071).

Strain	Growth medium	Incubation conditions	Control	Interpretation	Bile salt deconjugase activity
B. bifidum R0071	A RCM Agar plate supplemented with 0.5% (w/v) Taurodeoxycholic acid (TDCA)	Plates incubated for 5 days at 37°C under anaerobic conditions	Unsupplemented RCM agar plate	Bile salt deconjugase activity is manifest by the presence of clear precipitate halos around isolated colonies or opaque , granular white colonies compared to control colonies grown on unsup-plemented agar	Positive

6.3.4. Presence of Antibiotic Resistances Genes and Likelihood of transference

6.3.4.1. Minimal Inhibitory Concentrations

The generally recognized method to assess antibiotic susceptibility of microorganisms is by measuring the Minimal Inhibitory Concentration (MIC) and comparing it to standard microbiological breakpoints. Strains with MICs higher than the breakpoints are generally considered resistant. However, this result does not imply that the resistance can be transferred to other microorganisms.

Microbiological breakpoints were suggested by the FEEDAP Panel (European Food Safety Authority) for *Lactobacillus helveticus, Bifidobacterium infantis*, and *Bifidobacterium bifidum* in their "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veteran importance," published in June 2012[4]. This guidance document replaced the previous European Food Safety Agency (EFSA) opinion on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance, adopted in May 2005.

[4] This guidance document replaced the previous EFSA opinion on the updating of the criteria used in the assessment of bacteria of human or veteran importance, adopted on 18 June 2008.

The microbiological breakpoints were set for ten antimicrobial agents, which were chosen to maximize the identification of resistance genotypes to the most commonly used antimicrobials. It is mentioned in the guidance that the values should be reviewed on a regular basis and modified when necessary, as new data becomes available.

The MIC of several antimicrobial agents were determined for *L. helveticus* R0052, *B. longum* ssp. *infantis* R0033, and *B. bifidum* R0071 and compared with FEEDAP 2012 breakpoints.

The standard operational protocol (SOP) previously used by Lallemand Health Solutions (formerly known as Institut Rosell) was based on a compilation of various methods available at the time, for example ACE-ART 2005 and CLSI M100-S17 2007. The new SOP is based on the method recommended by ISO/IDF-ACE-ART 2009 on the basis of newly revised methodologies applied on determining MIC. The new SOP provides a more comprehensive approach and is in agreement with the new International standard protocol released in March 2009 by the International Organization for Standardization and International Dairy Federation (ISO/IDF).

- ***Lactobacillus helveticus* Rosell®-52 (R0052)**

For *L. helveticus* R0052, the MICs were determined by micro-dilution in LSM Broth using the Bio-Rad Plate reader. Strains with MICs higher than the breakpoints are considered as resistant.

Table 27. MIC for R0052 in LSM Broth Using the Recommended ISO/IDF Method.

Antimicrobial Agent	Minimal Inhibitory Concentration (µg/ml)	Microbiological breakpoints (µg/ml) *L. helveticus*
Amikacin	4	n.a.
Amoxicillin	0.125	n.a.
Amoxicillin-Clavulanic acid	8	n.a.
Ampicillin	<0.03125	1
Cefoxitin	4	n.a.
Ceftiofur	2	n.a.
Ceftriaxone	0.25	n.a.
Cephalothin	0.25	n.a.
Chloramphenicol	0.25	4
Ciprofloxacin	8	n.a.
Clindamycin	0.25	1
Erythromycin	0.125	1
Gentamicin[1]	1	16
Kanamycin[1]	4	16
Nalidixic acid	64	n.a.
Quinupristin/Dalfopristin	0.5	4
Streptomycin[1]	4	16
Sulfamethoxazole	64	n.a.
Tetracycline	0.125	4
Trimethoprim[1]	128	n.a.
Trimethoprim-Sulfamethoxazole	64	n.a.
Vancomycin	0.0625	2

[1]: possible interference of the growth medium
n.a.: not available

According to those breakpoints, *L. helveticus* R0052 is not considered resistant to any of the tested antimicrobial agents.

➢ ***Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)**

For *B. longum* ssp. *Infantis* R0033, the MICs were determined by micro-dilution in LSM Broth and cysteine using the Bio-Rad Plate reader.

Table 28 . MIC for R0033 in LSM Broth and Cysteine Using the ISO/IDF Recommended Protocol.

Antimicrobial Agent	Minimal Inhibitory Concentration (µg/ml)	Microbiological breakpoints* (µg/ml) *B. longum*
Amikacin	2	n.a.
Amoxicillin	<0.03125	n.a.
Amoxicillin-Clavulanic acid	<0.03125	n.a.
Ampicillin	<0.03125	2
Cefoxitin	0.5	n.a.
Ceftiofur	<0.03125	n.a.
Ceftriaxone	0.25	n.a.
Cephalothin	0.125	n.a.
Chloramphenicol	0.125	4
Ciprofloxacin	1	n.a.
Clindamycin	<0.03125	0.25
Erythromycin	<0.03125	0.5
Gentamicin[1]	8	64
Kanamycin[1]	16	n.r.
Nalidixic acid	16	n.a.
Quinupristin/Dalfopristin	0.25	1
Streptomycin[1]	<0.03125	128
Sulfamethoxazole	0.5	n.a.
Tetracycline	<0.03125	8
Trimetoprim	1	n.a.
Trimetoprim-Sulfamethoxazole	0.125	n.a.
Vancomycin	0.0625	2

[1]: possible interference of the growth medium

n.a.: not available; n.r.: not required

According to those breakpoints, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) is not considered resistant to any of the tested antimicrobial agents.

- ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

For *B. bifidum* R0071, the MICs were determined by micro-dilution in LSM Broth and cysteine using the Bio-Rad Plate reader.

Table 29. Minimal Inhibitory Concentration for R0071 in LSM Broth and Cysteine Using the ISO/IDF Recommended Protocol.

Antimicrobial Agent	Minimal Inhibitory Concentration (μg/ml)	Microbiological breakpoints (μg/ml) *B. bifidum*
Amikacin	0.5	n.a.
Amoxycillin	<0.03125	n.a.
Amoxycillin + Clavulanic acid	<0.03125	n.a.
Ampicillin	<0.03125	2
Cefoxitin	1	n.a.
Ceftriaxone	<0.03125	n.a.
Ceftiofur	<0.03125	n.a.
Cephalothin	1	n.a.
Chloramphenicol	0.125	4
Ciprofloxacin	8	n.a.
Clindamycin	<0.03125	0.25
Erythromycin	<0.03125	0.5
Gentamicin[1]	16	64
Kanamycin[1]	16	n.r.
Nalidixic acid	64	n.a.
Quinupristin/Dalfopristin	0.125	1
Streptomycin[1]	16	128
Sulfamethoxazole	2	n.a.
Tetracycline	0.25	8
Trimetoprim[1]	0.5	n.a.
Trimethoprim + Sulfamethoxazole	0.25	n.a.
Vancomycin	0.5	2

[1]: possible interference of the growth medium
n.r.: not required; n.a.: not available

According to those breakpoints, *Bifidobacterium bifidum* Rosell®-71 (R0071) is not considered resistant to any of the tested antimicrobial agents.

6.3.4.2. DNA Microarrays

In order to maximize the checking of the safety of the *L. helveticus* R0052, *B. longum* ssp. *infantis* R0033, and *B. bifidum* R0071, Lallemand Health Solutions obtained access to a microarray developed by Dr. Roland Brousseau, Group Leader of Environmental Genetics, and Dr Andre Nantel, Research Officer and head of the Microarray laboratory at the Biotechnology Research Institute (National Research Council of Canada, Montreal). This microarray allows detecting 166 known antibiotic resistance genes from each strain. This technique is faster and more reliable than the PCR techniques that were used in the past.

DNA oligonucleotides complementary to the sequence of known antibiotic resistance genes are generated and spotted onto specialized glass slides using specialized robots. Genomic DNA from the bacteria which are to be screened is first labeled with the fluorescent dye Cyanine-5 and then hybridized overnight to allow DNA to bind to complementary oligos. Upon excitation with fluorescent light, Cy5-labelled DNA which has hybridized to specific oligos will illuminate, allowing determination of the identity of the resistance gene.

Several recent studies have demonstrated the efficiency of this approach (Call et al. 2003; Frye et al. 2006; van Hoek et al. 2005), including one array designed specifically for the detection of antibiotic resistance genes (ABR) in LAB (Kastner et al. 2006). Recently, an array specific for >300 resistance genes was developed as part of the Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain (ACE-ART), a European-funded initiative with a mandate to determine the prevalence and risks posed by the presence of ABR genes in food-grade microorganisms.

The microarray used by Lallemand Health Solutions contained 182 oligonucleotides corresponding to 166 different acquired AMR genes targets (Garneau et al. 2010). EUB338-50 and EUB338-35 were included as positive controls for gram positive and gram negative bacteria, respectively while shuEUB-50 and shuEUB-35 were included as negative controls.

- ***Lactobacillus helveticus* Rosell®-52 (R0052)**

The screening for *L. helveticus* R0052 was negative on the ABR array and none of the tested antibiotic resistance genes were detected in the strain.

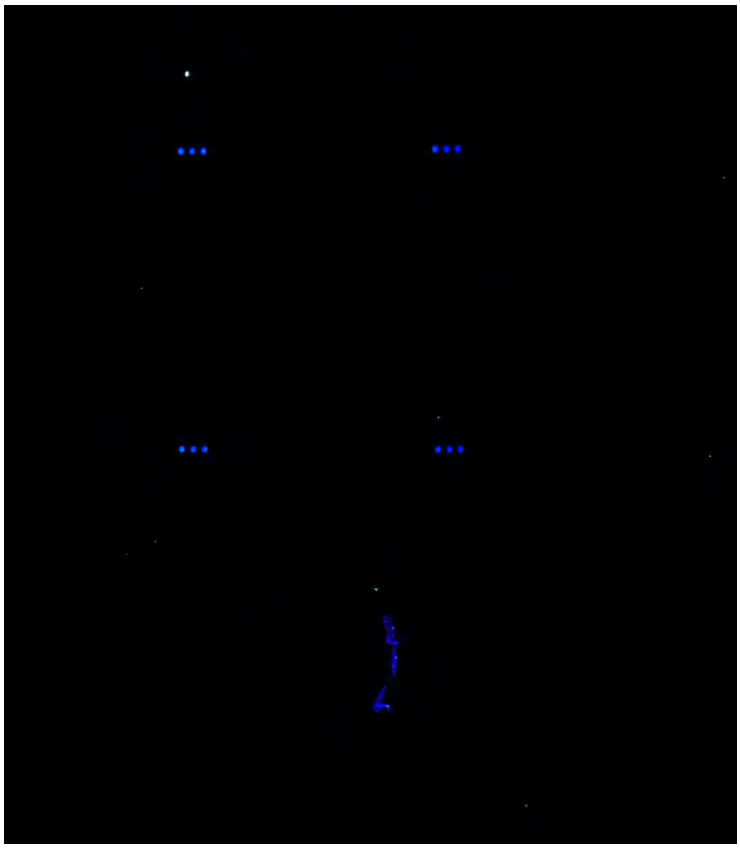


Figure 28. DNA Microarray of *L. helveticus* R0052 for the Detection of Antibiotic Resistance Genes.

These results provide evidence that the strain *L. helveticus* R0052 does not contain any of the tested ABR genes.

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033)

The screening for *B. longum* ssp. *infantis* Rosell®-33 (R0033) was negative on the ABR array and none of the tested antibiotic resistance genes were detected in the strain.

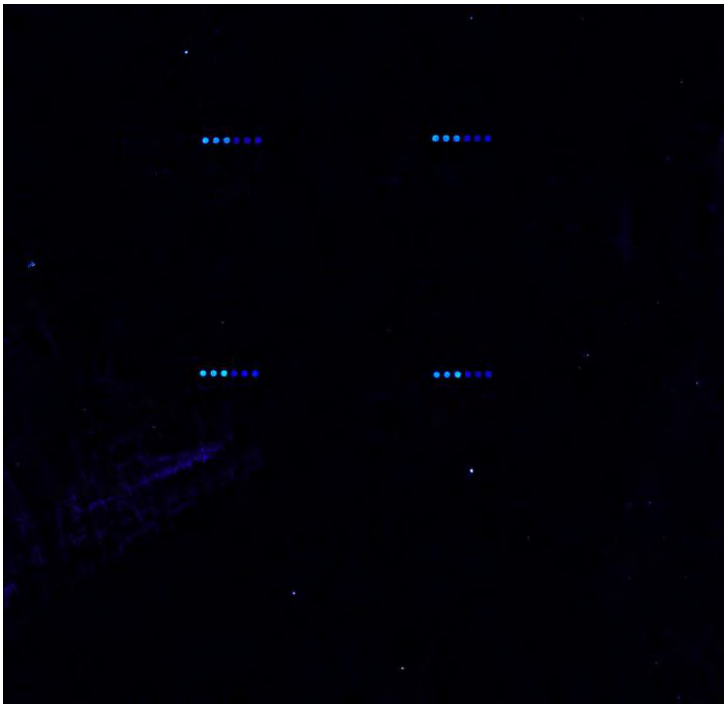


Figure 29. DNA Microarray of *B. longum* ssp. *infantis* R0033 for the Detection of Antibiotic Resistance Genes.

These results provide evidence that the strain *B. longum* ssp. *infantis* R0033 does not contain any of the tested ABR genes.

➢ ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

The screening for *B. bifidum* Rosell®-71 (R0071) was negative on the ABR array and none of the tested antibiotic resistance genes were detected in the strain.

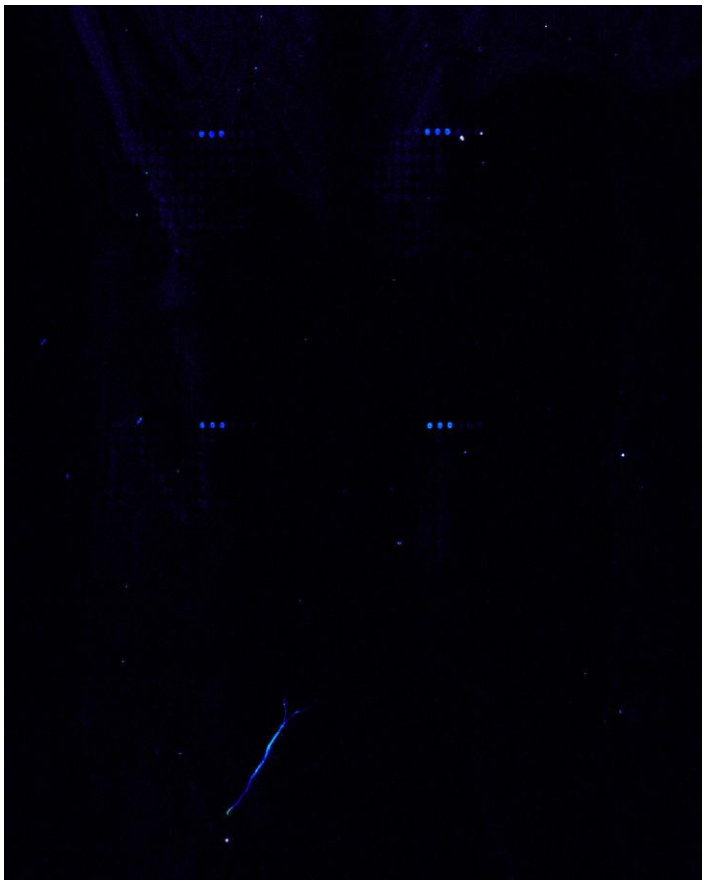


Figure 30. DNA Microarray of *B. bifidum* R0071 for the Detection of Antibiotic Resistance Genes.

These results provide evidence that the strain *B. bifidum* R0071 does not contain any of the tested ABR genes.

6.3.4.3. Antibiotic production

➢ ***Lactobacillus helveticus* Rosell®-52 (R0052)**

Lactobacilli are not known to be antibiotic producers. *L. helveticus* strain has not been reported in the literature as able to produce antibiotics. Moreover, the whole genome sequencing did not reveal any open-reading frames encoding genes for known antibiotic production. However, genes encoding putative bacteriocins were found. Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive bacterial species. *L. helveticus* R0052

was tested against a number of strains either by diffusion agar (Touré et al. 2003) or spot test (Yildirim 2001) and was shown not to possess any antimicrobial activity (Simard 2005; unpublished) against these microbes, therefore suggesting the genes encoding the bacteriocins may not be expressed.

Indicator strains	R0052 Supernatant
Staphylococcus saphrophyticus R0138	-
Staphylococcus aureus R0159	-
Salmonella enteriditis R0249	-
Salmonella typhimurium R0255	-
Enteroccus faecium R0074	-
Enteroccus faecium R0222	-
Bacillus cereus R0310	-
Bacillus cereus R0311	-
Bacillus subtilis R0179	-
Lactobacillus helveticus R0052	-
Lactobacillus plantarum R0202	-
Lactobacillus casei R0215	-
Pediococcus acidilactici R1001	-
Streptococcus thermophilus R0083	-

Moreover, whole genome sequencing has not revealed any open-reading frames encoding genes for antibiotic production.

- ***Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033)**

Bifidobacteria are not known to be antibiotic producers. *B. longum* ssp. *infantis* strain has not been reported in the literature as able to produce antibiotics. Moreover, whole genome sequencing has not revealed any open-reading frames encoding genes for antibiotic production.

- ***Bifidobacterium bifidum* Rosell®-71 (R0071)**

B. bifidum strain has not been reported in the literature as able to produce antibiotics. Moreover, whole genome sequencing has not revealed any open-reading frames encoding genes for antibiotic production.

6.4. In Vivo Studies

6.4.1. Studies in Infants and Children

6.4.1.1. Studies of the Three Individual Notified Probiotic Strains

A study was conducted to investigate the safety and tolerance of the three notified probiotic strains in infants aged 3–12 months (Manzano et al. 2017; Table 34). This was a multi-center randomized double-blind placebo-controlled 12 week trial with 4 treatment groups: *B. longum* ssp. *infantis* R0033, *L. helveticus* Rosell®-52 (R0052), *B. bifidum* Rosell®-71 (R0071), and placebo.

The probiotics were supplied as fine white powders packed in sealed sachets containing approximately $3x10^9$ cfu of *B. longum* ssp. *infantis* Rosell®-33 (R0033) or *L. helveticus* Rosell®-52 (R0052) or *B. bifidum* Rosell®-71 (R0071) freeze dried with potato starch as an excipient. The placebo was the same as the study product except that the sachet contained only the excipient, potato starch.

A total of 221 infants was recruited into the study. The inclusion criteria were “healthy infants aged between 3 and 12 months.” Of these, 202 (95 boys and 103 girls) completed the study and 19 subjects withdrew from the study for non-test-article related reasons. Two participants withdrew after two weeks of treatment due to constipation. The duration of the study for each participant was 12 weeks: 2-week run-in period, 8-week product-intake period, and 2-week follow-up period.

One of the study objectives was to investigate the safety and tolerance of these three probiotic strains, assessing a variety of safety and tolerance outcomes: anthropometric measures (weight, height, and head circumference), adverse events (including gastrointestinal symptoms, fever, and rashes), concentration of D-lactic acid, stooling characteristics (frequency, quantity, consistency, and color), and changes in sleep and crying patterns as general indicators of health status.

During the run-in period, each infant received a first visit to verify inclusion and exclusion criteria, perform the demographic profile, record subject characteristics and feeding option, and obtain relevant medical history as well as measure weight, length, and head circumference.

After the run-in, infants were randomized to receive *B. longum* ssp. *infantis* Rosell®-33 (n=51), *L. helveticus* Rosell®-52 (n=50), *B. bifidum* Rosell®-71 (n=49), or placebo (n=52) once daily for 8 weeks. Each sachet was diluted in 10 mL warm water, breast milk, or infant formula.

Data from the 202 randomized infants were used for the intention-to-treat (ITT) analysis. These infants had completed all 12 weeks of the study protocol. Data from 198 randomized infants were used for the per-protocol (PP) analysis with 4 participants excluded as protocol violators.
The statistical analysis of the data related to growth parameters (length, weight, and head circumference) showed that all 4 groups (probiotic strains and placebo) started the study at visit 1 with similar weight, height, and head circumference (Table 30). Growth among the four groups did not differ significantly (Table 31).

Table 30. Anthropometric Measures of the Participants at the Beginning of the Study.

	B. infantis R0033	*L. helveticus* R0052	*B. bifidum* R0071	Placebo	*P*-value
Height (cm)	66.50 (62.25-71.00)	68.50 (64.25-71.00)	67.00 (65.00-71.00)	67.25 (63.87-70.00)	0.765 [c]
Weight (Kg)	7.44 ± 1.28	7.58 ± 1.13	7.59 ± 0.98	7.55 ± 1.22	0.914 [d]
Head circumference (cm)	43.00 (41.60-44.75)	43.75 (42.00-45.00)	43.00 (42.00-44.00)	43.25 (41.50-44.12)	0.834 [c]

[c] Kruskal-Wallis test
[d] ANOVA test.

Table 31 . Evaluation of Growth (Height, Weight and Head Circumference) of the Participants During the Intervention Period.

	B. infantis R0033	*L. helveticus* R0052	*B. bifidum* R0071	Placebo
Treatment (56th day)				
Height (cm)	2.50 (2.00-3.40) [a]	2.60 (2.00-3.45) [a]	3.00 (2.00-3.80) [a]	3.00 (2.00-4.00)
Weight (Kg)	0.79 (0.57-0.90) [a]	0.71 (0.49-0.90) [a]	0.63 (0.46-0.80) [a]	0.70 (0.56-0.98)
Head circumference (cm)	1.00 (1.00-1.85) [a]	1.00 (1.00-2.00) [a]	1.00 (1.00-2.00) [a]	1.00 (1.00-2.00)

The change between visit 2 and 3 was expressed as median (25Q - 75Q) for the recorded outcome.
[a] Significant equivalence with a 95% confidence level ($p < 0.05$) when compared with the placebo group using a non-parametric tost-test.

Adverse events were recorded throughout the study from the start of product intake (starting after visit 2) until the final visit (8 weeks study product + 2 weeks follow-up period [visit 4]). None of the participants suffered from a serious adverse event (SAE) during the study (Table 32).

For the PP population, significant equivalence to the placebo was observed in all 3 groups. All adverse events were related to gastrointestinal disorders (Table 32).

For the ITT population, only the *B. infantis* R0033 group showed a total number of AEs not equivalent to that found in the placebo group ($p \leq 0.085$). This non-equivalence was caused by the 4 infants who were protocol violators in that they did not take the study products as required and were withdrawn from the study.

Indeed, it was due specifically to the high number of AEs (n=9) registered by one of the participants of the *B. infantis* R0033 group who was noncompliant with the product intake: 2 gastrointestinal; 4 respiratory, thoracic, and mediastinal; 1 eye and 1 skin and subcutaneous tissue disorders; and 1 episode of fever. None of these AEs was related to product intake. Further, such non-equivalence was not detected for the number of participants with at least one AE ($P \leq 0.001$) or the number of “possibly related” AEs (Table 32).

Table 32. Adverse Events for All Participants That Completed the Study Classified According to the System Organ Class (SOC) of the MedDRA.

	Number of AEs				Number of participants with at least 1 AE			
Body System	**R0033 (N=48)**	**R0052 (N=50)**	**R0071 (N=49)**	**Placebo (N=51)**	**R0033 (N=48)**	**R0052 (N=50)**	**R0071 (N=49)**	**Placebo (N=51)**
Gastrointestinal disorder	24	16	15	13	19	14	13	13
Infections and infestations	2	2	6	5	2	2	6	3
Bone and joint injuries	0	4	1	4	0	4	1	4
Nervous system disorders	0	0	0	1	0	0	0	1
Respiratory,thoracic and mediastinal disorders	19	14	10	9	13	9	7	7
Skin and subcutaneous tissue disorders	0	2	0	2	0	2	0	2
Eye disorders	4	2	2	2	2	2	2	2
Ear and labyrinth disorders	3	3	3	1	3	2	3	1
Fever (LLT)	7	7	2	5	6	5	2	5
Total	57[a]	50[a]	39[a]	42	45[a]	40[a]	34[a]	38

Body System	Number of "Possibly-Related" AEs				Number of participants with at least 1 "Possibly-Related" AEs			
	R0033 (N=48)	R0052 (N=50)	R0071 (N=49)	Placebo (N=51)	R0033 (N=48)	R0052 (N=50)	R0071 (N=49)	Placebo (N=51)
Gastrointestinal disorder	3	3	1	1	3	3	1	1
Infections and infestations	0	0	0	0	0	0	0	0
Bone and joint injuries	0	0	0	0	0	0	0	0
Nervous system disorders	0	0	0	0	0	0	0	0
Respiratory,thoracic and mediastinal disorders	0	1	0	0	0	1	0	0
Skin and subcutaneous tissue disorders	0	0	0	0	0	0	0	0
Eye disorders	0	0	0	0	0	0	0	0
Ear and labyrinth disorders	0	0	0	0	0	0	0	0
Fever (LLT)	0	0	0	0	0	0	0	0
Total	3	4	1	1	3	4	1	1

R0033= *Bifidobacterium longum* subsp. *infantis* R0033; R0052= *Lactobacillus helveticus* R0052; R0071= *Bifidobacterium bifidum* R0071; LLT = low level term

[a]Significant equivalence with a 95% confidence level ($p < 0.05$)

The adverse events recorded as gastrointestinal symptoms, fever, or rashes were collected throughout the study period (from visit 2 until visit 4) during clinic visits or phone calls or in the daily diaries.

The results showed a low incidence in all of these safety variables for all 4 groups of the study (Table 33). Additionally, the proportion of affirmative answers was below the statistical biological difference which is used for the 10% for success variables, with yes/no answers.

Nevertheless, although the equivalence analysis could not be done, the behavior of the treatment groups was similar to that observed for the control group. These results were also cross-checked with the daily questionnaires completed by the parents. No discordance was found between both sources of data (data not shown).

Concerning changes in sleep and crying patterns, the incidence of these indicators was also below the statistical biological difference of the 10% and was homogenous among all study groups; see

Table 33 below (Da Silva et al. 2008).

Table 33. Summary of Affirmative Answers to Safety Control Questions.

	Absolute Frequencies (Relative Frequencies)			
	B. infantis R0033 (N=459)	*L. helveticus* R0052 (N=450)	*B. bifidum* R0071 (N=441)	Placebo (N=468)
Fever	14 (0.032)	20 (0.044)	13 (0.029)	12 (0.027)
Rash	5 (0.012)	6 (0.013)	5 (0.011)	3 (0.007)
Diarrhea	22 (0.051)	13 (0.029)	13 (0.029)	14 (0.032)
Unscheduled visit to doctor	32 (0.074)	31 (0.069)	21 (0.048)	25 (0.057)
Change in sleeping habits	47 (0.109)	61 (0.036)	41 (0.088)	39 (0.088)
Crying	53 (0.123)	80 (0.178)	67 (0.143)	59 (0.134)

Questions asked at the weekly phone calls and at visit 3 and 4:
Fever: Did your infant suffer a fever episode in the last week?
Rash: Did your infant have a rash on any part of his/her body in the last week?
Diarrhea: Did your infant have diarrhea in the last week?
Unscheduled visit to doctor: Did your infant have an unscheduled visit to the doctor last week? Why?
Change in sleeping habits: Were there any changes in your infant's sleeping habits?
Crying: Did your infant cry excessively in the last week?

All measures of the concentration of D-lactic in urine samples were below the quantification limit of the method (33 µM) for all test samples.

Stooling frequency and stool consistency, amount, and color were recorded daily by the infants' parents using the Amsterdam Stool Chart, which enables parents and clinicians to rate different aspects of stools of both premature and term infants. Statistical analysis showed that the infants' stooling characteristics (frequency, quantity, consistency, and color) in each of the three probiotic groups were equivalent to those of the placebo group.

The use of medication during the study by the infants in each of the 4 groups was very low and similar in all 4 groups.

In conclusion, the consumption of *B. longum* ssp. *infantis* R0033, *L. helveticus* R0052, *B. bifidum* R0071 was well tolerated and safe for infants from 3 to 12 months of age at a dose of 3 billion cfu per day.

Table 34. Study of Three Probiotic Strains: *B. longum* ssp. *infantis* (R0033), *L. helveticus* (R0052), and *B. bifidum* (R0071).

References	Objectives	Study Design	Subjects	Strains Dose/Day	Duration	Safety-Related Results
Manzano et al. (2017)	Investigate the safety and tolerance of three probiotic strains *B. longum* ssp. *infantis* Rosell®-33 (R0033); *L. helveticus* Rosell®-52 (R0052) and *B. bifidum* Rosell®-71 (R0071)	Multi center randomized double-blind placebo-controlled 12 week study with 4 treatment groups; *B. longum* ssp. *infantis* Rosell®-33 (R0033); *L. helveticus* Rosell®-52 (R0052); *B. bifidum* Rosell®-71 (R0071) and placebo	202 infants (3-12 months)	$3x10^9$ cfu	12 Weeks	The data related to the primary outcome, growth, showed that all participants grew similarly independent of the group where they were allocated. Regarding safety variables, none of the participants suffered a Serious Adverse Event during the study and all groups were equivalent in the number of Adverse Events. The number of episodes of fever and the number of unscheduled visits to the doctor were equivalent in all groups of the study. None of the participating infants showed any signs of D-lactic acidosis. The changes in sleeping and crying habits show that all 4 groups were homogenous in their responses. The consumption of *B. longum ssp. infantis* R0033, *L. helveticus* R0052, B. *bifidum* R0071 was well tolerated and safe for infants from 3 to 12 months of age, at a dose of 3 billion cfu per day.

6.4.1.2. Studies of Probiokid®

Probiokid® is a blend of the three specific probiotic strains *L. helveticus* Rosell®-52 (80%), *B. longum* ssp. *infantis* Rosell®-33 *(*10%), and *B. bifidum* Rosell®-71 (10%), the same proportions as are intended for addition to infant formula powder. While Probiokid® contains, in addition to the probiotic strains, potato starch, vanilla flavor, and fructooligosaccharides, published studies in which Probiokid® is consumed at levels providing $3x10^9$ or $5x10^9$ cfu/day of the three strains provide evidence of the safety of ingestion of these strains.

Three studies (Wang 2012, Gao 2013, and Wu 2013) were conducted in infants diagnosed with non-infectious diarrhea. These studies were conducted in hospitalized infants from 0 to 36 months with non-infectious diarrhea due to climate and food intolerances/allergies, etc. according to the national standards described in the diagnosis and treatment strategies of diarrhea in China (detailed further in Fang et al. 1998). The current recommended practice for treatment of non-infectious diarrhea in China is the administration of Smecta®, a dioctahedral montmorillonite suspension that acts primarily by adsorbing water and thereby reducing free stool water (Szajewska et al. 2006). Smecta® was used as an active control in the three studies.

Wang (2012) studied the effectiveness of Smecta® and Probiokid® versus Smecta® alone in 194 infants aged 3-36 months diagnosed with non-infectious diarrhea, randomized into two groups. The dose of the synbiotic and smectite given to the infant depended on the age (sub-groups: < 1year; 1-2 years; 2-3 years). Efficacy was evaluated according to the Chinese national standards described in Fang et al. (1998). “Markedly Effective” was the frequency and characteristics of the stool returning to normal (<3 bowel movements per day) and symptoms disappearing within 72 hours of treatment; “Effective” was significant improvement of the frequency (<4 bowel movements per day) and characteristics of stool and symptoms lessening within 72 hours of treatment; “Ineffective” was no improvement of the frequency and characteristics of stool and no improvement in symptoms within 72 hours of treatment.

The results showed that the total effective rate (Markedly Effective + Effective) in the combined Smecta® + Probiokid® group was 90-93% (depending the age sub-group) versus Smecta® alone, which was 87-88% effective. The difference was not significant by chi-squared analysis. However, when looking just at Markedly Effective rates, the Smecta® + synbiotic was significantly better than Smecta® only for each age sub-group ($p \leq 0.05$ by χ^2 test). The author concluded that the combination of Probiokid® and Smecta® should be used routinely in the treatment of non-infectious diarrhea. There were no reports of adverse events.

Gao (2013) and Wu (2013) ran studies very similar to that described by Wang (2012) in 86 infants aged 0-36 months and 148 infants aged 2-36 months. They found the total effective rate was significantly improved in the combination Smecta® + Probiokid® (approximately >90% effective in both studies in all age-subgroups) compared to the Smecta® only group (between 63-79% effective depending on age group and study). They also concluded that Probiokid® is safe and effective and should be included in the treatment of infantile diarrhea. No adverse reactions were reported in either group in the Gao (2013) study; Wu (2013) did not indicate occurrence of any adverse events.

Three additional studies (Cui and Wure 2003, Mei and Chen 2008, and Yang et al. 2010) were conducted in children with diarrhea due to rotavirus infection. These studies were conducted in infants from one month up to 5 years of age in accordance with the Chinese national standards for diarrhea diagnosis and treatment described in Fang et al. (1998). Rotavirus was confirmed by antigen detection in the stool using an enzyme-link immunosorbent assay (ELISA).

Cui and Wure (2003) studied 122 infants aged 6-24 months with positive fecal rotavirus antigen who had diarrhea for less than three days at the time of admittance. The infants were randomized into two groups: Probiokid® (sold at that time under the name Biostime) – 62 participants or *Lactobacillus* spp. (Lacidophilin) – 60 participants. In addition, both groups received Ribavirin therapy. (Ribavirin is a guanosine analog which acts by blocking viral RNA synthesis and is typically used to treat severe respiratory infections, hepatitis C and viral hemorrhagic fevers.) The duration of diarrhea in the Probiokid® group was 39.3±17.1 hours while that in the Lacidophilin group was 63.8±22.9 hours. In the Probiokid® group, there were 45 Markedly Effective cases, 13 Effective cases and 4 Ineffective cases. The total effective rate was 93.5% (58/62). In comparison, in the Lacidophilin group there were 12 Markedly Effective cases, 25 Effective cases and 23 Ineffective cases and the total effective rate was 61.7% (37/60). The difference was statistically significant ($p \leq 0.01$). There was no report of adverse events.

In Mei and Chen (2008), 78 infants aged 0-5 years with confirmed rotavirus infection were randomized into two arms. Probiokid® was used in conjunction with intravenous Ribavirin therapy and compared to a control arm that received only the Ribavirin therapy. The primary objective of the study was to determine if Probiokid® could enhance the effectiveness of the anti-virus therapy. Efficacy was assessed according to the Chinese national standards (Fang et al. 1998) as described above. In the Ribavirin control group, (39 patients): Markedly Effective in 13 cases; Effective in 16 cases; Ineffective in 10 cases. The effective rate was 74.3%. In the Probiokid® treatment group (39 patients): Markedly Effective 19 cases; Effective in 18 cases, Ineffective in 2 cases. The effective rate was 94.9%. The difference in treatment efficiency between the two groups was statistically significant ($p \leq 0.05$). The authors concluded that the combined use of Probiokid® and Ribavirin showed a better effect in relieving pediatric diarrhea caused by rotavirus infection. No adverse events were reported.

Yang et al. (2010) randomized 98 infants aged 6 to 30 months with confirmed rotavirus infection into two arms. The 58 cases in the treatment arm received Probiokid® and lactose-free milk formula while the control arm (40 cases) received either milk formula or were breast-fed. Both groups continued to receive comprehensive therapy (fluid therapy; bacteria-infected patients were administered with the same antibiotics; orally administered intestinal mucous protection agents such as Smecta®; proper supplement of Vitamin B, Vitamin C, folic acid and other supporting treatments). The primary objective was to determine if the combination of lactose-free milk and Probiokid® could impact the duration of the symptoms. The efficacy was assessed in a similar manner described previously. The duration of diarrhea was significantly reduced (2.8±1.1 days vs. 4.9±2.6 days, $p \leq 0.01$) as was the duration of hospital stay (5.5±1.7 days vs 8.5±2.3 days, $p \leq 0.01$). Clinically effective rate in the treatment group was 94.8% vs 77.5% in the control group ($p \leq 0.05$). The authors concluded that the combination of lactose-free milk

powder and Probiokid® could be useful in the clinic for the supplemental treatment of infantile autumn diarrhea caused by rotavirus. No adverse events were reported.

Two studies (Chen et al. 2007 and Pantovic 2013) examined the impact of Probiokid® on IgA levels. The design of these studies was not well described and lacked elements associated with Good Clinical Practice (GCP).

Chen et al. (2007) examined 28 healthy children in four different age groups (<1 year, 1-2 years; 2-3 years; 3-4 years) having low secretory IgA levels in their saliva (IgA2 predominant). The children were given Probiokid® twice a day for 13 days; saliva samples were taken on day 0, 1, 4, 7, 11 and 14. The control group was composed of 8 aged-matched children without any intervention during the same period. There were improvements in sIgA concentrations for the individuals in all age groups taking Probiokid® relative to the age-matched controls. The authors claimed the changes in salivary sIgA were significant and stated that Probiokid® was effective in helping to achieve and maintain normal levels of sIgA. There was no report of adverse events.

Pantovic (2013), in an open-label study, examined the effect of Probiokid® on serum IgA (IgA1 predominant) over a six-month period. The 31 children in this study were 6-42 months of age and had been hospitalized for respiratory and/or ear infections. They were chosen for this study because they also had low levels of immunoglobulins, particularly IgA. They were further stratified by atopic status (high, low, and non-atopic) established by allergy skin prick test to 25 allergens. Sample size was determined by a power calculation and the serum IgA levels of the participants were compared to themselves at baseline, 3 months, and 6 months. Participants received one sachet of Probiokid® per day for the entire period of the study. The author found that the IgA levels normalized in 35% of the children after 3 months and 81% after 6 months and that by 3 months of supplementation there was clinical improvement in the respiratory/ear infections. The author concluded that Probiokid® supplementation can lead to clinical improvement of infection in less than 3 months but 6 months of supplementation are required to increase serum IgA levels. No adverse effects were reported.

Two further studies (Cazzola et al. 2010b and Stojkovic et al. 2016) investigated the prevention of common winter diseases and respiratory infections. Cazzola et al. (2010b) described a randomized, double-blind, placebo-controlled study investigating the effect of daily supplementation with Probiokid® for 3 months. The study was conducted in young school-age children (2 to 7 years old at enrollment) during a winter period. Participants were otherwise healthy children who suffered from at least three episodes of ear, nose, throat (ENT), respiratory tract, or gastrointestinal (GI) illness, diagnosed by their physician, during the previous winter. The multicenter study was conducted in France between December 2006 and March 2007 and was performed according to current French and European regulations.

One hundred and thirty-five healthy school-age children were randomized to receive either Probiokid® [1 sachet per day containing $3x10^9$ cfu of bacteria, and 750 mg of FOS] or placebo supplementation for 3 months. The treatments were presented in similar sachets with similar looking contents and flavor to be taken once daily. Parents were given a diary and were instructed to note any health problems (including the nature of the problem and major symptoms, duration of the episode, daily body

temperature during the episode, any over-the-counter treatments given, and duration of any school-day loss) which occurred during the course of the study. The children were examined by investigators at 28, 56, and 84 days and the parents' diaries were checked for completeness.

During the study timeframe, 135 children (63 girls and 72 boys) were eligible for inclusion in the study (Intention-To-Treat [ITT] population). The mean age was 4.1±1.1 years (median: 4.0 years; range: 2-7 years). Sixty-two children were allocated to receive Probiokid® and 73 children received the placebo. Nineteen children in the treated group (30.6%) and 15 children in the placebo group (20.5%) prematurely stopped the allocated treatment; the main reasons being the occurrence of an intercurrent health problem or a non-study-related intercurrent event.

A total of 82 children out of 135 reported at least one health event during the study course; 50 out of 73 in the placebo group (68.5%) and 32 out of 62 in the treated group (51.6%). The difference between the two groups accounts for a 24.7% relative risk reduction ($p \leq 0.045$) in the treatment group.

This difference was due to a decrease in the number of children who suffered from at least one ENT, respiratory tract, or GI disorder (50.0% with treatment group versus 67.1% with placebo; $P \leq 0.044$).

Moreover, at least one sickness school day loss was noted in 42.5% of children in the placebo group, as compared with 25.8% in the Probiokid® group, corresponding to an improvement of 40% ($P \leq 0.043$).

A total of 126 patients (58 and 68 patients in the placebo and Probiokid® group, respectively) received either treatment for at least 14 days (Per-Protocol [PP] population). A health event occurred in half of the treated group (53.4%) and in a majority of the placebo group (72.1%) with a significant difference. The difference between the two groups accounts for a 25.8% relative risk reduction ($p \leq 0.031$) in the treatment group.

Results are summarized in the following table:

Children with at least one episode		Placebo group	Probiokid® group	RRR	*p*-value
Any symptoms (%)	ITT	68.5	51.6	24.7%	0.045
	PP	72.1	53.4	25.8%	0.031
At least one ENT, respiratory or digestive symptoms (%)		67.1	50.0	25.5%	0.044
At least one school day loss		42.5	25.8	39.3%	0.043

One hundred and fifty-one health events (57.6% in the placebo group and 42.4% in the Probiokid® group) were reported by 82 children. The respiratory tract and ENT accounted for the majority of these events (76.2%) while 15.9% of events were digestive.

Overall, 67.1% of children in the placebo group and 50.0% of children in the Probiokid® group suffered from at least one health event involving ENT or GI symptoms. Additionally, there was a difference in the number of school days lost to sickness with

the treatment group at 25.8% as compared to 42.5% for the placebo group with a significant difference of $p \leq 0.043$.

In the present study, the principal outcome was the percentage of children who suffered from at least one health problem during the course of the study. A secondary outcome was the percentage of children with at least one health problem involving one or more days of school lost. Between the placebo and treatment groups, the study detected a statistically significant 25% relative risk reduction in the percentage of children who suffered from at least one health event during the winter period. Furthermore, in the treatment group, the episodes also appeared to be less severe than those of the placebo group as evidenced by the overall reduction in the number of school days lost.

This pilot study, conducted in otherwise healthy 2-7 year-old children with previous acute respiratory or gastrointestinal episodes during winter, documented that a 3-month supplementation with the Probiokid® decreased the risk of occurrence of common infectious diseases and limited the risk of school day loss.

Adverse events:
Investigators reported a total of 24 adverse events in 20 children (Table 35). None were serious events. Most of these events were expected ENT, respiratory tract, or GI problems. In two cases (abdominal pain in the placebo group and an otitis media in the treatment group) the intensity of the event was noted as severe. Two adverse events with digestive problems were considered by investigators as possibly related to the study medication in the placebo group and none in the Probiokid® group.

Table 35. Adverse Events Reported by Investigators (Cazzola et al. 2010b).

	Placebo group (n=73)	Treatment group (n=62)	Total (n=135)
Number of children with at least one adverse event (%)	9 (12.3%)	11 (17.7%)	20 (14.8%)
Nature of the adverse event			
Digestive problem	5	1	6
Varicella	2	3	5
Dysuria	1	1	2
Flu-like symptoms	1	1	2
Adenoidectomy	0	1	1
Ankle edema	0	1	1
Ankle sprain	0	1	1
Eczema	1	0	1
Laryngitis	0	1	1
Leg pain	0	1	1
Otitis	0	1	1
Throat ache	0	1	1
Topical Allergy	0	1	1
Total	10	14	24

In an open-label study, Stojkovic et al. (2016) assessed the optimal time of use of probiotics and prebiotics in controlling respiratory infections and wheezing in young children based on the analysis of 78 hospitalized children aged 1.5 months to 5 years. The children were classified into 3 groups: Group I - with respiratory infection and wheezing; Group II - with respiratory infection without wheezing; Group III - with wheezing but without accompanying respiratory infection. The children were given a dietary supplement synbiotic (Probiokid®) containing $5x10^9$ cfu [combination of *L. helveticus* Rosell®-52 (R0052) (80%), *B. longum* ssp. *infantis* Rosell®-33 (R0033) (10%), and *B. bifidum* Rosell®-71 (R0071) (10%)] and 750 mg FOS for a period of 9 months. Data were recorded for each of the groups on the incidence of respiratory infections and wheezing during the 9 month supplementation period.

The results showed that after a 3 month supplementation with the synbiotic, children in Groups I and II, who usually suffered from episodes of pneumonia, had a statistically significant decrease in the incidence of respiratory infections. This improved state was maintained after 6 and 9 month supplementations (Table 36). This confirms the study by Cazzola et al. (2010b), who found a lower incidence of respiratory infections in school age children after a 3 month supplementation with the synbiotic.

Table 36 . Frequency of Respiratory Infections in Children During Probiokid® Supplementation (Stojkovic et al. 2016).

Period	Group of patients		
	I	II	III
Baseline	62.7%	70.6%	36.4%
After three months	7.8%	5.9%	9.1%
After six months	0.0%	0.0%	0.0%
After nine months	0.0%	0.0%	0.0%

The statistically significantly decreased incidence of respiratory infections was also followed by a falling incidence of concomitant wheezing in children in Groups I and II (Table 37).

Table 37. Frequency of Wheezing in Children during Probiokid® Supplementation (Stojkovic et al. 2016).

Period	Group of patients		
	I	II	III
Baseline	92.0%	41.2%	100.0%
After three months	35.3%	5.9%	54.5%
After six months	0.0%	0.0%	0.0%
After nine months	0.0%	0.0%	0.0%

Serum levels of IgA were measured before the administration of the synbiotic and found to be in deficit in 18% of the study patients. After a 3-month supplementation, a statistically significant increase in serum IgA levels was observed in patients in all 3 groups and continued to rise after 6 and 9 months of supplementation. This rise in

serum levels in children from Groups I and II was followed by an improvement of clinical symptoms. Children in Group III also exhibited a statistically significant rise in serum IgA levels but the increase occurred at a slower pace, only within 6-9 months supplementation, and coincided with a statistically significant decrease in episodes of wheezing.

This study showed that Probiokid® is effective for immunomodulation in children. The optimal duration of administration was found to be 3 months to provide effective control of the frequency of respiratory infection. At least 6 months administration was required to reduce the frequency of episodes of wheezing. No side effects of Probiokid® were identified in the examined children and it was well tolerated in children aged 1½ months to 5 years.

Two additional minor studies (Jiang 2008 and Xi et al. 2013) have been performed with Probiokid®. Jiang (2008) studied a pediatric population (52 cases aged 3-24 months) with persistent diarrhea of undefined etiology. This was a two-arm study; one group received the synbiotic (n=32) and the other a probiotic described as “Golden Bifido” (n=20) having 1/100th the dose of bacteria compared to the synbiotic (i.e. 0.05 billion vs 5 billion cfu per dose). Conventional treatment, including oral administration of digestive medicines, mucosal protective agents, oral rehydration salts, or intravenous rehydration, was provided according to the degree of dehydration. Probiokid® normalized the number of defecations per day within 6 days of treatment. The treatment time and cost significantly favored Probiokid® when compared to the probiotic as did the total clinical effective rate, calculated as 91% vs 65% ($p \leq 0.01$). The authors concluded that Probiokid® should be used with conventional therapy to improve the cure rate. There was no report of adverse events.

In the second study, Xi et al. (2013) examined the impact of Probiokid® on oral thrush in 70 children aged 1-26 months. The children were randomized into two groups of 35, and received either 2% sodium bicarbonate with nystatin or the same plus Probiokid®. The effective rate (94.3% vs 77.1%, $p \leq 0.05$) and the reoccurrence rate (2.9% vs 17.1%, $p \leq 0.01$) significantly favored the Probiokid® arm. The authors concluded that Probiokid® can be effective in the treatment of oral thrush in children when used with nystatin and sodium bicarbonate.

Table 38. Studies of *Probiokid*® in Infants and Children.

References	Objectives	Study Design	Subject	Dose/Day	Duration	Safety-Related results
Cui and Wure (2003)	Evaluate Biostime (Probiokid®) for the treatment of 62 cases of pediatric rotavirus gastroenteritis	Randomized, controlled Biostime (Probiokid®) group (n=62) Age <12mo: 5 B cfu QD; Age 12-24 mo: 5B cfu BID Lacidophilin group (n=60) Both groups also received Ribavirin. Intervention continued until diarrhea resolved	122 children (6-24 mo) who had diarrhea for less than 3 days and who tested positive for Rotavirus antigen in their feces	<12 months: 5 billion cfu/day; 12-24 months: 10 billion cfu/day	Not stated (but evaluated treatment effects for at least 72 h)	Biostime (Probiokid®) group duration of diarrhea was 39.3±17.1h while that in the Lacidophilin group was 63.8±22.9h. Biostime (Probiokid®) group: the total effective rate is 93.5% (58/62). Lacidophilin group, the total effective rate was 61.7% (37/60). The difference was significantly different ($p \leq 0.01$). There was no report of adverse events.
Chen et al. (2007)	Evaluate the impact of the symbiotic on IgA level	Randomized, controlled 1 Biostime (Probiokid®) sachet (5B cfu), BID, for 13 days	28 children less than 4 years, divided into 4 groups by age. Control was 2 children from each age group (8 children)	10 billion cfu/day	13 Days	For the children who had low sIgA level before taking the sachet, the sIgA level increased to and was maintained at a normal level after they took the sachets. No adverse events were reported.
Jiang (2008)	Clinical evaluation of Biostime (Probiokid®) in the treatment of children with persistent diarrhea	Randomized, active control Biostime (Probiokid®) group (n=32) <6mo: 2.5B cfu BID; 6-12 mo: 5B cfu BID; 12-24 mo: 5-10B cfu BID Golden Bifido group (n=20). Intervention continued until diarrhea resolved	52 children (3 to 24 mo) in hospital or outpatient clinic with persistent diarrhea	<6 months: 5 billion cfu/day; 6-12 months: 10 billion cfu/day; 12-24 months: 10-20 billion cfu/day	Treated until diarrhea resolved	After 6 days of treatment, Biostime (Probiokid®) group had normalized the number of defecations per day whereas the Golden Bifido group remained high ($p \leq 0.05$). Treatment time (7.1 vs 12.6 days) and cost (652 vs 843 Yuan) was significantly ($P \leq 0.001$) better in Biostime (Probiokid®) group compared to Golden Bifido. Clinically effective rate in Biostime (Probiokid®) was 91% vs 65% ($p \leq 0.01$). No adverse events were reported.

Mei and Chen (2008)	Evaluate the therapeutic effect of Biostime (Probiokid®) product on pediatric diarrhea caused by rotavirus infection	Randomized, active control Biostime (Probiokid®) group: (n=39) 1 Biostime (Probiokid®) sachet (5B cfu) BID, for 7 days + Ribaviren Control group: (n=39) Ribaviren only	78 children (0-5 yrs) with rotavirus infection	10 billion cfu/day	7 days	Difference in treatment effective rate between the two groups was significant, in favor of the probiotic group (94.9 vs. 74.3%; p≤0.05). There was no report of adverse events.
Cazzola et al. (2010b)	Investigate the effects of a synbiotic supplementation in reducing common winter diseases in children	Double-blind, randomized, placebo controlled Probiokid® group (n=62): 3B cfu/day for 3 months, Placebo group (n=73)	135 school-age children (3 to 7 years old); suffered at least 3 physician diagnosed episodes of ENT, respiratory or GI infection last winter	3 billion cfu/day	3 months	Decrease in the % of children who suffered from at least one health problem during the 3-month study compared with placebo. Relative risk reduction is 24.7% (P≤0.045) Decrease in the % of children suffering from at least one episode characterized by an ear, nose and throat (ENT), respiratory tract or gastrointestinal symptom compared with placebo (50% vs. 67.1% ; P≤0.044) Decrease in the % of children with at least one health problem including one or more day school loss compared with placebo (25.8% vs. 42.5%; P≤0.043). Investigators reported a total of 24 adverse events in 20 children. None were serious events. Most of these events were expected ENT, respiratory tract or gastrointestinal problems. In two cases the intensity of the event was noted as severe. Two adverse events with digestive problems were considered by investigators as possibly related to the study medication in the placebo group and none in the Probiokid® group.

Yang et al. (2010)	Observe the therapeutic effects of supplemental feeding with lactose-free milk powder combined with Biostime (Probiokid®) on the infantile diarrhea	Randomized, controlled Biostime (Probiokid®) Group: (n=58) 1 Biostime (Probiokid®) sachet (5B cfu) + lactose-free milk powder formula. Control group (n=40) + breast-fed or formula fed.	98 infants (6-30 mo) admitted to inpatient clinic between Jan 2008-Oct 2009 with diarrhea due to rotavirus infection	5 billion cfu/day	Not stated	Significant improvement ($p \leq 0.01$) in the disappearance of diarrhea symptom (2.8±1.1 days vs. 4.9±2.6 days) and duration of hospital stay (5.5±1.7 days vs 8.5±2.3 days). Clinically effective rate in Biostime (Probiokid®) was 94.8% vs 77.5% ($p \leq 0.05$, analyzed by χ^2 test). No adverse events were reported.
Wang et al. (2012)	Evaluate the effectiveness of Smecta and the synbiotic versus Smecta alone in infants diagnosed with non-infectious diarrhea	Randomized, active control Observation group (n=104): oral Smecta + Biostime (Probiokid®) <12 mo (n=33): 1.7B cfu TID 13-24 mo (n=43): 2.5 B cfu BID 25-36 mo (n=28): 5B cfu BID Active Control (n=90): oral Smecta only 0-12 mo (n=31); 13-24 mo (n=35): 25-36 mo (n=24). For 3 days.	194 children (aged 3-36 months) with non-infectious diarrhea	<12 months: 5 billion cfu/day 13-24 months: 5 billion cfu/day 25-36 months: 10 billion cfu/day	3 days	No adverse reactions. Analyzed by χ^2 test. Observation group effective rate was 90.7-92.9% vs control group effective rate of 87.1-88.6% (Not significant). However, very effective rates are statistically significant: 78.8-82.1% for treatment group vs. 74.2-75% for control. ($p \leq 0.05$). There was no report of adverse events.
Gao (2013)	Evaluate the effectiveness of Smecta and the synbiotic versus Smecta alone in infants diagnosed with non-infectious diarrhea	Randomized, active control Observation group (n=43): oral Smecta + Biostime (Probiokid®) 0-12 mo: 1.7B cfu TID 13-24 mo: 2.5B cfu BID 25--36 mo: 5B cfu TID Active Control (n=43): oral Smecta only	86 hospitalized children (0-36 mo) with non-infectious diarrhea	0-12 months: 5 billion cfu/day 13-24 months: 5 billion cfu/day 25-36 months: 15 billion cfu/day	3 days	No adverse reactions. Analyzed by χ^2 test. Observation group effective rate was 90.7% vs control group effective rate of 62.8% ($p \leq 0.05$). No adverse reactions were observed in either group.

Pantovic (2013)	Investigate the effectiveness and the optimal time of supplementation with synbiotic in atopic children with common respiratory and/or ear infections	Uncontrolled before and after study 3B cfu/day for 6 months	31 atopic children (6 to 42 mo) hospitalized with common respiratory and/or ear infections and low sIgA.	3 billion cfu/day	6 months	After 3 months level of IgA increased for 1.8 times up from 0.33±3.42 g/l to 0.6±0.78 in 35% children and after 6 months increased for 3.9 times up to 1.3±1.76 in 81% children (t=0.43, $p \leq 0.05$). At least 6 months is the optimal duration of supplementation with synbiotic to reduce the risk of common infectious disease. No adverse events were reported.
Wu (2013)	Evaluate the effectiveness of Smecta and the synbiotic versus Smecta alone in infants diagnosed with non-infectious diarrhea	Randomized, active control Observation group (n=84): oral Smecta + Biostime (Probiokid®) <12 mo (n=32): 1.7B cfu TID 13-24 mo (n=35): 2.5 B cfu BID 25-36 mo (n=17): 5B cfu BID Active Control (n=64): oral Smecta only 0-12 mo (n=21); 13-24 mo (n=33): 25-36 mo (n=10).	148 hospitalized children (2-36 mo) with non-infectious diarrhea.	<12 months: 5 billion cfu/day 13-24 months: 5 billion cfu/day 25-36 months: 10 billion cfu/day	3 days	Analyzed by χ^2 test. Intervention group had a significantly more effective rate that the control for all groups. <12 mo: 93.8% vs 76.1% 13-24 mo: 91.4% vs 78.8% 25-36 mo: 82.3% vs 60.0% $p \leq 0.05$ in all groups There was no report of adverse events.
Xi et al. (2013)	Examine the impact of the symbiotic on oral thrush	Randomized, active control Experimental group (n=35): 2% sodium bicarb + nystatin + 1 sachet Biostime (Probiokid®) (5B cfu) BID Active Control (n=35): 2% sodium bicarb + nystatin. After 3 days, effective rate. For 14 days, follow up after 30 days for recurrence rate.	70 children (42M/28F; aged 1-36 mo) diagnosed with oral thrush.	10 billion cfu/day	17 days	No adverse reaction were reported. Experimental group vs Control group: Total effective rate: 94.3% vs 77.1%, $p \leq 0.05$ Recurrence rate: 2.9% vs 17.1%, $p \leq 0.01$

Stojkovic et al. (2016)	Determine optimal time efficiency of a synbiotic in controlling respiratory infections and wheezing disease	Children were classified into 3 groups; Group I - with respiratory infection and wheezing; Group II - with respiratory infection without wheezing; Group III - with wheezing but without accompanying respiratory infection. No control group	78 children (1.5 months to 5 years)	5 billion cfu/day	9 months	Synbiotic is effective for immunomodulation, controlling frequency of respiratory infections by 3 months and wheezing by 6 months. No side effects of synbiotic were identified in the examined children and it was well tolerated.

6.4.1.3. Studies of *L. helveticus* Rosell®-52 (R0052) in Other Formulations

L. helveticus Rosell®-52 (R0052) has been extensively marketed by Lallemand Health Solutions for use in infants and children as a part of the following blends:

- *Lacidofil*®, a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus* Rosell®-52 (R0052) (5%). Each capsule of Lacidofil® contains $2x10^9$ cfu, corresponding to 10^8 cfu of *Lactobacillus helveticus* Rosell®-52 (R0052).
- Oralis SB® powder, a combination of *L. helveticus* Rosell®-52 (R0052) (54%), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%), and *S. cerevisiae* var *boulardii (*7%*).* Each sachet of Oralis SB® powder contains $1.25x10^9$ cfu of bacteria, corresponding to $6.7x10^8$ cfu of *L. helveticus* Rosell®-52 (R0052).

The research studies of products containing *L. helveticus* Rosell®-52 discussed below are summarized in Table 39 at the end of this section.

- ***Clinical Studies of Lacidofil®***

A study was conducted by Ivanko et al. (2005; unpublished) with the objective of studying the frequency of diarrhea associated with intensive antibiotic treatment in children, the capability of clostridial toxins A and B to induce diarrhea, and assessment of a possible protective effect produced by Lacidofil®. Fifty-seven children (35 boys and 22 girls) aged 1 to 16 years were randomly divided into two groups. The patients were suffering from severe diseases which required an intensive, long-term treatment with antibiotics. Thirty-one children suffered from a complicated form of pneumonia, 10 patients had burn disease, 6 had surgical pathology of the abdominal cavity during the postoperative period, 5 had complicated fractures of extremities, and 5 had pyelonephritis. All children were checked for presence of toxin A+B in the feces. Concentration of toxins in the feces of 50 µg/ml and greater were considered clinically significant and capable of causing *Clostridium difficile* associated enteropathy.

The 27 children in the experimental group were taking from 1 to 6 Lacidofil® capsules per day, depending on age, simultaneously with the antibiotic treatment, for 2 weeks to 2 months. The control group of 30 children took the antibiotics without the probiotic. Diarrhea was observed in 7.4% of children in the experimental group and 36.7% of children in the control group. The presence of clostridial toxins found in the feces was 7.4% and 43.3%; $p \leq 0.01$. It was suggested that *L. helveticus* affects the capability of *C. difficile* to form enteropathogenic and cytopathogenic toxins in the bowel. There was no report of adverse events.

A randomized, controlled study by Marushko et al. (2007) found Lacidofil® reduced the side effects of antibiotic treatment in 34 children (10 months to 3 years old) with acute broncho-pulmonary pathology (bronchitises and pneumonias). Patients in the Lacidofil® group (n=16) received, in addition to their antibacterial therapy, $2x10^9$ cfu/day when

aged under 1 year old and $4x10^9$ cfu/day when older than one year. Patients in the control group received the antibacterial therapy alone.

Antibiotic-associated diarrhea (AAD) occurred in 2 of 16 participants (12.6%) receiving Lacidofil®, compared to 8 of 18 (44.8%) in the control group ($p \leq 0.05$). In addition, the duration of diarrhea was significantly shorter in the treatment group (2.6 ± 1.1 days) compared to the control group (5.9 ± 1.2 days) ($p \leq 0.05$). No side effects resulting from the use of Lacidofil® were recorded, and the treatment was well tolerated by the children.

Aryayev and Konenko (2009) randomized 36 children from 3 months to 18 years old with upper respiratory infection who received antibacterial therapy in addition to their classical treatment. Eighteen patients were included in the first group, receiving Lacidofil® in addition to their basic therapy and antibiotic treatment, while the 18 patients in the control group received only their basic therapy and antibiotic treatment.

Daily dosage of Lacidofil® was $2x10^9$ cfu/day for children under 12 months, $4x10^9$ cfu/day for children from 1 to 3 years old, $6x10^9$ cfu/day for children from 3 to 12 years old, and $12x10^9$ cfu/day for children over 12 years old. Monitoring of stool frequency and consistency was assessed through a diary filled by the patients. The percentage of AAD episodes was statistically significantly lower in the Lacidofil® group (5.5%) than in the control group (28.9%), corresponding to a relative risk reduction of 80% for AAD with Lacidofil®.

The safety and tolerance of the preparation were evaluated according to presence or absence of side effects, including allergic reactions and cases of individual intolerance. No such side effects were observed during the treatment with Lacidofil®.

Maydannik et al. (2010) conducted a randomized, controlled study of 214 children being treated with antibiotics for respiratory, urinary, or digestive illnesses. The children receiving Lacidofil® (n=117, 1-3 capsules/day, $2x10^9$ cfu/capsule, for 14-21 days) experienced a 1.5-fold lower incidence of AAD, and a two-fold decrease in the duration of diarrhea (2.9 days vs 5.9 days, $p \leq 0.05$). *C. difficile* A and B toxins in children from the Lacidofil® group were reduced from 16.2% to 2.5% after 3 weeks, corresponding to a statistically significant 84.5% decrease of *C. difficile.* No side effects were recorded during the study.

A randomized, controlled study by Pastera et al. (2010) found that Lacidofil® reduced *C. difficile* carriage in children with pulmonary tuberculosis. The efficiency of Lacidofil® administration was studied for 55 children aged 3-18 years, after 4 to 5 months of therapy, by clinical data (temperature, no coughing, and appetite), bacteriological data, and X-ray study.

Children were divided into two groups: the control group (n=36) received the conventional antituberculosis chemotherapy (i.e. antibiotics) and the main group (n=23) received, with the chemotherapy, the Lacidofil® probiotic preparation at doses of 6×10^9

cfu/day or 12×10^9 cfu/day for one month.

Patients treated with conventional therapy and the probiotic at a dose of 6×10^9 cfu/day for one month experienced a significant increase in the amount of *C. difficile* toxins A and B in the stool (from 1.1±0.2 ng/ml to 2.4±0.4 ng/ml, $p\leq0.05$), whereas patients treated with conventional therapy and probiotics at a dose of 12×10^9 cfu/day for one month experienced a significant reduction of toxins A + B in stool (from 1.53±0.3 ng/ml to 0.93±0.1 ng /ml, $p\leq0.01$). Additionally, patients on Lacidofil® experienced improved appetite, normalized stool, reduced gastrointestinal discomfort, and the disappearance of glossitis during the course of treatment. No adverse events were reported.

Another post-market clinical study (Wasowska-Krolikoeska 1997; unpublished) confirmed the efficacy of the Lacidofil® preparation in preventing AAD in pediatric populations when given concurrently with the antibiotic treatment (i.e., cephalosporins and aminoglycosides). It was found that Lacidofil®, when given to infants and children during treatment with cephalosporins and aminoglycoside antibiotics, made maintenance of normal intestinal flora possible. No difficulties with treatment or drug intolerances were reported.

Hegar et al. (2015) performed a prospective, randomized, double-blind, placebo-controlled trial to investigate the efficacy of probiotics added to oral rehydration solution (ORS) and zinc in the treatment of acute infectious diarrhea and moderate dehydration in children aged 6 - 36 months. A total of 112 children was divided into two groups. Each group consisted of 56 subjects. Patients were given ORS (Indoralite®) *ad libitum* and 20 mg zinc sulphate/day for 10 days and Lacidofil® or placebo capsules for 7 days. Lacidofil® did not shorten the duration of diarrhea in comparison to standard therapy with ORS and zinc. The median daily frequency of defecation until diarrhea stopped was 5.0 in the supplemented vs. 5.5 in the control group ($P\leq0.795$). No adverse effects were reported.

Patients being treated with antibiotics for *Helicobacter pylori* infections may also benefit from Lacidofil®. Gnaytenko et al. (2009) investigated the effects of Lacidofil® in 45 children from 6 to 16 years old receiving anti-helicobacter therapy. Children given Lacidofil® ($4x10^9$ cfu/day for 20 days) in combination with amoxicillin and clarithromycin had a significantly lower incidence of AAD (8.0±5.5%) compared to patients treated without probiotics (35.0±10.9%; $p\leq0.05$). Additionally, *C. difficile* toxins were found in the stool of five children (25%) treated without probiotics, but only in one (4.0%) child given Lacidofil® ($p\leq0.05$). Thus, administration of Lacidofil® from the first day of anti-helicobacter therapy reduced AAD and prevented colonization of the digestive tract by toxin-forming *C. difficile*. There was no report of adverse events.

Abaturov et al. (2014) reported a clinical study to evaluate the efficiency of Lacidofil® in the treatment of *Helicobacter pylori* infection in children. The study involved 45 children aged 10 to 16 years with active chronic *H. pylori*-associated gastroduodenitis, which

were randomized into the following groups: Treatment group (n=25), which received the anti-*Helicobacter pylori* eradication therapy with Lacidofil®. Control group (n=20), which received only the anti-*Helicobacter* therapy. Lacidofil® was administered at a dose of 1 capsule, three times daily after the meals, for 21 days. The total dose per day was $6x10^9$ cfu.

Efficiency of the treatment was assessed 6 weeks after anti-*Helicobacter* therapy, on dynamics of clinical parameters and safety of treatment ("Helpil"-test; serum sCD14 levels; expression of NF-KB by peripheral CD40+ cells). In addition, clinical symptoms of pain before and during treatment of patients were also evaluated using a 4-point scale.

In the Lacidofil® group, there was a more rapid regression of the intensity of the pain syndrome, dyspeptic syndrome, and astheno-vegetative syndrome. Furthermore, only two of the children in the probiotic group demonstrated antibiotic-associated intestinal lesions, while antibiotic-associated intestinal lesions were reported in 1/3 of the patients in the control group. Lacidofil® also produced an increase in sCD14, which is associated with decreased activity of NF-κB. *S*ignificantly higher *H. pylori* eradication was achieved by anti-*Helicobacter pylori* therapy with supplemental use of Lacidofil® compared to control (96% vs. 70%, $p \leq 0.03$). No adverse events were reported.

Two studies by Tlaskal et al. in 1995 and 2005 showed that hospitalized children supplemented with Lacidofil® showed a reduction in pathogens involved in gastroenteritis and recovered faster from diarrhea related to various pathogens when compared to control groups.

In Tlaskal et al. (1995), supplementation was shown to be effective against *Citrobacter freundii.* In this proactive controlled clinical study of 75 pediatric patients suffering from diarrhea, 33 children were given $2\text{-}6x10^9$ cfu/day of Lacidofil® for 10-14 days (though 3 patients received Lacidofil® treatment for 1-3 months), while 42 patients were treated by conventional treatments involving mixtures of absorption clay, smectite (Smecta pulvis) and a concentrate of metabolic products of common intestinal symbiotic bacteria (preparation Hylak forte). Otherwise, treatment in the two groups was the same and included a regimented diet.

Oral administration of Lacidofil® relieved diarrhea symptoms (i.e., quality and quantity of stool) in patients with acute gastroenteritis more quickly than the conventional treatment (3.1 days vs. 6.8 days, $p \leq 0.01$). The incidence of *Citrobacter freundii* decreased, particularly in infants. Culture findings from stool examinations before and after treatment show *Citrobacter freundii* remained positive in 5 of 12 children. Complete resolution of clinical conditions of patients with gastrointestinal tract diseases were also noted to occur significantly sooner for those treated with Lacidofil® as compared to the conventional treatment (4.8 days vs. 8.7 days, $p \leq 0.01$).

Since the patients were not randomly assigned to the treatment and control groups, but rather were assigned to the Lacidofil® treatment according to the greater severity of their condition, it was noted that the quicker resolution of their symptoms was all the more significant. There was no report of adverse events.

In Tlaskal et al. (2005), a randomized, double-blind trial was implemented at pediatric outpatient departments in Prague on 113 children 1 to 6 years of age with uncomplicated acute diarrheal disease. The diet regimen was used as a basic treatment of the disease and the children received either a placebo or Lacidofil® ($2x10^9$ cfu/day), or the metabolites.

Electron microscopy demonstrated the viral etiology of the disease in 62.8% of patients; 48.7% of all the diarrheas were induced with rotaviruses, which were still present in the stools after 10 days of the treatment in 18.6% of patients. Lacidofil® significantly reduced the time necessary for adjustment of the stool consistency (5.45±2.33 days for placebo, 4.0±2.02 days for Lacidofil®, 6.14 ± 3.2 days for metabolites) but no change in the stool frequency was demonstrated ($p \leq 0.03$). There were no statistically significant differences between values of stool IgA and saliva IgA among the treatment methods used. There was no report of adverse events.

Skorodumova et al. (2007) conducted a controlled study to evaluate the effectiveness of Lacidofil® in children with acute intestinal infections. A total of 248 patients with mild and moderate forms of acute intestinal infection aged 6 months to 1 year were divided into two groups. In the probiotics group, 1 capsule of Lacidofil® was administered 3 times daily with meals or within 30 minutes after the meal prior the termination of symptoms. Before the treatment with Lacidofil®, all patients of the probiotic group underwent microbiological examination of the colon, which revealed an imbalance of microflora in all children. During the treatment, the examination of microflora showed a restoration of the number of *lactobacilli* and *bifidobacteria*, indicating that Lacidofil® promoted more rapid recovery of normal intestinal flora. Additionally, the microflora also showed significantly decreased *Escherichia coli* load (less than 10^6). No adverse events were reported. Hegar et al. (2013) conducted a randomized, double-blind, placebo controlled, parallel group study with 70 children aged ≥ 5 years seen mainly because of complaints of epigastric pain. They received omeprazole (Proton Pump Inhibitors, PPI) 20 mg/day and Lacidofil® (n=36) or placebo (n=34) for 4 weeks. The objective was to evaluate the incidence of small bowel bacterial overgrowth in children treated with omeprazole, and to test whether probiotics influenced the incidence. No significant difference was detected between groups regarding the incidence of positive breath tests 33% vs 26.5%, $p \leq 0.13$, suggesting that Lacidofil® had no effect on the risk of developing small bowel bacterial overgrowth SBBO during PPI therapy.

Children suffering from lactose intolerance may also benefit from Lacidofil®. A randomized single-blind two-arm clinical trial was conducted by Rampengan et al. (2010) to assess the efficacy of live versus killed probiotics in children with lactose

malabsorption using the breath hydrogen test. Seventy-nine lactose intolerant children 10-12 years old were randomly divided into 2 groups to receive either live or killed probiotic. The live probiotic group took 1 capsule of Lacidofil® daily for 2 weeks and the killed probiotic group took 2 sachets of Dialac daily for 2 weeks. For the probiotics group, the total dose per day was $2x10^9$ cfu. Children were followed up to determine if they developed any symptoms or adverse reactions.

The mean breath hydrogen test score before administration of live probiotic was 34.5, which decreased significantly to 22.1 ($p \leq 0.001$) at 120 minutes after administration of live probiotic. In the killed probiotic group, the mean breath hydrogen test score before administration of the killed probiotic was 36.0, decreasing significantly to 20.3 ($p \leq 0.001$) at 120 minutes after administration of killed probiotic. The difference between the live and killed probiotic groups at 120 minutes after ingestion of lactose was not significantly different.

This study also showed that symptoms linked to lactose intolerance were improved (e.g., frequent flatus, bloating, nausea, vomiting, abdominal pain, abdomen distention and diarrhea). Indeed, before the treatment with live and killed probiotics, 55 children had abdominal pain, 11 were asymptomatic, and 10 had more than one symptom, while at the end of treatment, 46 children were asymptomatic, 23 had abdominal pain, and 5 had more than one symptom. These changes were statistically significant. During the study period, there were no side effects reported in children receiving live or killed probiotic.

A placebo-controlled, clinical trial was conducted by Chernyshov (2009) to investigate clinical and immunologic effects of Lacidofil® in the treatment of children with atopic dermatitis and allergy to cow's milk. A total of 58 children aged 2 months to 4 years were randomized into 2 groups:

- Probiotic group: 30 children received 1 capsule of Lacidofil® daily for 30 days at a daily dose of $2x10^9$ cfu.
- Placebo group: 28 children received 1 capsule daily of placebo (maltodextrin) for 30 days.

After the treatment, a significantly marked reduction of SCORAD was reported in 63.3% of patients in the probiotic group and 32.1% in the placebo group ($p \leq 0.02$). During the follow-up period, three children from the probiotic group and nine children from the placebo group had to use topical steroids at least once. The decline of SCORAD in patients who did not use topical steroids during the follow-up period was significant in the probiotic group ($p \leq 0.01$) and was not significant in the placebo group. After the treatment, levels of specific IgG4 to cow's milk increased statistically significantly and percentages of transitional recently activated CD45RA+RO+T cells decreased statistically significantly. The significant increase of specific IgG4 levels in the probiotic

group that was not reported in the control group could be considered a shift to tolerance. There was no report of adverse events.

- ***Clinical Studies on Oralis SB®***

Oralis SB® is a combination of *L. helveticus* Rosell®-52 (R0052) (54 %), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%) and *S. cerevisiae* var *boulardii (7%).* Each sachet of Oralis SB® powder contains $1.25x10^9$ cfu of bacteria, corresponding to $6.7x10^8$ cfu of *L. helveticus* Rosell®-52 (R0052). The studies described in this section were conducted in India, where Oralis SB® is commercialized under the brand name Darolac.

Jindal et al. (2011) conducted a clinical study in children aged 7-14 years with Oralis SB® to explore the effect of oral probiotics on salivary *Streptococcus mutans* counts and thus to evaluate if oral probiotics are a preventive tool against the development of tooth decay. The prospective investigation utilized a randomized, double-blind, placebo-controlled study design in which 150 healthy children, with no history of antibiotic intake or topical fluoride therapy for the preceding month, were randomly allocated among three groups: Group A (n=50)--placebo, Group B (n=50)--Oralis SB®, or Group C (n=50)--Sporolac (a product containing $1.5x10^8$ spores of *Bacillus coagulans)*. Sachets were taken twice daily, once in the morning and once one hour after lunch, for 14 days. To evaluate the effect of oral probiotic on salivary *S. mutans* counts, saliva samples were collected at baseline and one day after the 14 days of intervention. Daily consumption of both probiotics for two weeks statistically significantly reduced the salivary levels of *S. mutans* colony counts. No adverse events were reported.

Thakkar et al. (2013) conducted a clinical study to compare the effect of oral use of the probiotic preparation Oralis SB® with the effect of chlorhexidine mouth rinses on dental plaque accumulation. In a randomized double-blind three-arm trial, 90 children aged 13-15 years, having mild to moderate or good to fair plaque scores, and with no active carious lesions and no recent antibiotic therapy (within 4 weeks), were randomly allocated into three intervention groups: Group 1 (n=30)--placebo, Group 2 (n=30)--Chlorhexidine, and Group 3 (n=30)--Oralis SB®. According to the results of plaque scores, there was a statistically significant difference between groups on the 15th day ($p \leq 0.001$) and a statistically significant difference between groups after 3 weeks ($p \leq 0.012$), but not at baseline ($p \leq 0.431$). When the chlorhexidine and probiotic groups were compared, the plaque scores were statistically significantly less in the probiotic group both on the 15^{th} day of intervention and 3 weeks after discontinuation of intervention. There was no report of adverse events.

A double-blind randomized controlled trial was conducted by Puranaik et al. (2014) to investigate if Oralis SB® was effective in reducing plaque and gingivitis among adolescents aged 15-16 years. Ninety healthy adolescents were allocated to Group A (n=30), receiving Chlorhexidine gluconate, Group B (n=30), receiving Oralis SB®, or Group C (n=30), receiving placebo mouth rinse. Each subject received one test product for 14 days. At the end of treatment there was a statistically significant inhibition ($p \leq 0.05$) of plaque accumulation and of gingival scores, for the chlorhexidine and probiotic groups, but not for the placebo group. Additionally, the probiotic mouth rinse was significantly more effective on gingival scores than the chlorhexidine mouth rinse at

day 14 ($p \leq 0.01$). No side effects were reported in this study by the examiner responsible for conducting the enquiry on adverse events.

Table 39. Studies with *L. helveticus* Rosell®-52 Used in Other Formulations.

Infants and children studies of Lacidofil®, a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus* Rosell®-52 (R0052) (5%). Each capsule of Lacidofil® contains 2x10^9 cfu, corresponding to 10^8 cfu of *L. helveticus* Rosell®-52 (R0052).						
References	**Objectives**	**Study Design**	**Subject**	***L. helveticus* Dose/Day**	**Duration**	**Safety-Related results**
Tlaskal et al. (1995)	Efficacy of Lacidofil® in pediatric patients with disease of the gastrointestinal tract	Non-randomized controlled clinical trial 2 groups: control group (n=42) / Lacidofil® (2x10^9 cfu) group (n=33) 1 at 3 capsules/days for 10-14 days and for 3 patients for 1-3 months	75 pediatric patients with acute intestinal infection	0.1 billion cfu at 0.3 billion cfu/day	1-3 months	Lacidofil® relieved diarrhea symptoms (i.e., quality and quantity of stool) more quickly than the conventional treatment. There was no report of adverse events.
Wasowska-krolikoeska (1997; unpublished)	Prevention and treatment of intestinal dysbiosis	Uncontrolled. Age < 3 years: ½ -1 capsule, TID, orally Age < 15 years: 1-2 capsules, TID, orally	20 children and infants (up to age 15 years)	< 3years: 0.15 to 0.3 billion cfu/day >3 years: 0.3 to0.6 billion cfu/day	14-21 days	Prophylactic Lacidofil® maintained normal stool and microflora. Lacidofil® was effective in the maintenance of normal intestinal microflora during treatment with antibiotics (i.e., cephalosporins and aminoglycosides) and treating dysbacteriosis resulting from antibiotics. No difficulties with treatment or drug intolerances.
Ivanko et al. (2005; unpublished)	Prevention of AAD	Randomized, controlled Control (n=30) / Lacidofil® (n=27) 1 to 6 capsules/day (depending on age)	57 children (12 months – 16 years) taking long-term antibiotics	0.1 to 0.6 billion cfu/day	2 weeks – 2 months (depending on age)	Decreased number of diarrhea cases in probiotic group compared to control (7.4% vs 43.3%, p≤0.01). No adverse events were reported.
Tlaskal et al. (2005)	Efficacy on children's diarrhea	Randomized, double blind, controlled study 3 groups: placebo group / Hylac ® group / Lacidofil® (2x10^9 cfu) group 1 capsule/day for 10 days	113 pediat-ric patients (1-6 years) with acute diarrhea (gastro-enteritis)	0.1 billion cfu/day	10 days	Lacidofil® significantly reduced the time necessary for adjustment of the stool consistency compared to placebo. (4 vs. 5.45 days, p≤ 0.05) and Hylac (4 vs. 6.14, p≤0.003). There was no report of adverse events.

Marushko et al. (2007)	Prevention of AAD	Randomized controlled clinical trial 2 groups: control group (n=18) / Lacidofil® ($2x10^9$ cfu) group (n=16) age <1 year: 1 capsule/day, age 1-3 years: 1 capsule bid, for 2-4 weeks	34 children with respiratory pathologies and under antibiotics (10 months – 3 years)	<1years: 0.1 billion cfu/day 1-3 years: 0.2 billion cfu/day	2-4 weeks	• In control group, diarrhea was observed more frequently than in the probiotic group (44.8% vs. 12.6% $p \leq 0.05$). • The duration of diarrhea was significantly lower in children taking Lacidofil® (2.6±1.10 days) than in the control group (5.9±1.16 days, $p \leq 0.05$). • In Lacidofil® group, there was a significant improvement of the intestinal microflora. No side effects resulting from the use of Lacidofil® were recorded, and the treatment was well tolerated by the children
Skorodumova et al. (2007)	Evaluate the effectiveness of Lacidofil® in children with acute intestinal infection	Controlled clinical trial 2 groups: control group (225) / Lacidofil® ($2x10^9$ cfu) group (23) 1 capsules TID , for 14 days	248 pediatric patients with acute intestinal infection (6 to 12 months)	0.3 billion cfu/day	14 days	Lacidofil® significantly restored intestinal microbiome, improved immune status (increases phagocytosis) and significantly increased IgA/IgG. No adverse events were reported.
Aryayev and Konenko (2009)	Efficiency and safety of Lacidofil® ®in prevention of AAD	Randomized controlled clinical trial 2 groups: control group (n=18)/ Lacidofil® ($2x10^9$ cfu) group (n=18) age <1 year: 1 capsule/day, 1-3 years: 1 caps BID , 3-12 years: 1 caps TID, >12 years: 2 caps TID	36 children with muco-viscidosis and under antibiotics (3 months -18 years)	<1years: 0.1 billion cfu/day 1-3 years: 0.2 billion cfu/day 3-12 years: 0.3 billion cfu/day >12 years: 0.6 billion cfu/day	2 Months	There were statistically ignificantly fewer episodes of AAD in the probiotic group than the control group (5.5% vs. 28.9%). 80% risk reduction of AAD when Lacidofil® was added to antibiotic treatment. No side effect was reported in this study.

Chernyshov (2009)	Clinical and immunologic effects of probiotics in the treatment of children with atopic dermatitis	Randomized, placebo-controlled study Lacidofil® (n=30), 1 capsule/day Placebo (n=28)(malto-dextrin), one capsule/day	58 children (2 mo to 4 years) with atopic dermatitis and allergic to cow's milk	0.1 billion cfu/day	30 days	Patients with marked decline of clinical signs higher in the probiotic group than placebo (63.3% vs. 32.1%, p≤0.02). Significantly reduced use of topical steroids in probiotic group only (p≤0.01). There was no report of adverse events.
Gnaytenko et al. (2009)	Study AAD frequency in children who were receiving AHT (antihelicobacter therapy) and efficiency of its prevention with Lacidofil®	Controlled 2 groups with AHT: Control group and Probiotic group: 1 capsule of Lacidofil® BID for 20 days	45 children (age 6 to 16 years) undergoing treatment for *H. pylori*-positive disease forms of the gastroduodenal area	0.2 billion cfu/day	20 days	Incidence of AAD was significantly less frequent in probiotic group compared to control (8.0% vs. 35.0%, p≤0.05), and no cases of enterocolitis were diagnosed with the use of Lacidofil® from the first day of Anti-helicobacter therapy. No adverse events were reported.
Maydannik et al. (2010)	Efficacy and safety of Lacidofil® in treatment and prevention of AAD for children	Randomized, controlled 2 groups: control group (n=127) / Lacidofil® ($2x10^9$ cfu) group (n=117) age <1 year: 1 capsule/day, 1-3 years: 1 capsule BID, 3-12 years: 1 capsule BID or tid, >12 years: 1 or 2 capsule TID for 14-21 days.	244 children with acute respiratory, urinary or digestive infections and under antibiotics (0–17 years)	<1years: 0.1 billion cfu/day 1-3 years: 0.2 billion cfu/day 3-12 years: 0.2 to0.3 billion cfu/day >12 years: 0.3 to 0.6 billion cfu/day	14-21 days	*C. difficile* eradicating effect of Lacidofil® has been recorded for 84.5% of children carrying toxins (vs. no eradication in control group) and no reported side effects. Compared to controls, children receiving Lacidofil® also experienced a 1.5-fold lower incidence of AAD and a two-fold decrease in duration of diarrhea.(2.9 vs. 5.9 days, p≤0.05) No side effect has been reported in this study.
Pastera et al. (2010)	Preventive and therapeutic effect of	Randomized controlled clinical trial 3 groups: control group	• 59 children with pulmonary	Group A: 0.3 billion cfu/day Group B: 0.6	1 month	At a dose of $12×10^9$ cfu/day for one month caused a significant reduction of toxins A + B in stool.

	Lacidofil® for the rifampicin-associated intestinal infection *C. difficile* & the safety during long term chemotherapy	(n=36) / Lacidofil® ($2x10^9$ cfu) group (n=23) Group A: 3 capsules /day Group B: 6 capsules /day	tuberculosis and under antibiotics (3-18 years)	billion cfu/day		(p≤0.01). Patients on Lacidofil® experienced significantly improved appetite, normalized stool, reduced gastrointestinal discomfort, and the disap-pearance of glossitis. There was no report of adverse events.
Rampengan et al. (2010)	Assess live versus killed probiotics in children with lactose malabsorption	Single blind, randomized, comparison study 2 groups: killed probiotic group (Dialac) (n=39)/ Lacidofil® ($2x10^9$ cfu) group (n=40) 1 capsule daily	• 79 lactose intolerant children (10-12 years)	0.1 billion cfu/day	2 weeks	In both groups with probiotic (killed and live), there was a significantly decrease of BHT (breath hydrogen test) (p≤0.001), and an improving of the Lactose tolerance. During the study period, there were no side effects reported in children receiving live or killed probiotic
Hegar et al. (2013)	In children treated with omezaprole, incidence of small bowel bacterial overgrowth (SBBO), and influence of probiotics on incidence	Double-blinded, placebo-controlled 2 groups: Placebo (n=34) / Lacidofil® ($2x10^9$ cfu) (n=36) 1 capsule/day	70 children (ages 6-17)	0.1 billion cfu/day	4 weeks	No statistically significant differences occurred in incidence of positive breath tests for SBBO between treatments (33% vs. 26.5%, p≤0.13).
Hegar et al. (2015)	Investigate the efficacy of probiotics added to oral rehydration solution (ORS) and zinc in the treatment of acute infectious diarrhea and moderate dehydration	Prospective randomized double blind placebo controlled trial. 2 groups: Placebo (n=56)/ Lacidofil® ($2x10^9$ cfu) (n=56) daily	112 children (6-36 months)	0.1 billion cfu/day	7 days	No side effects have been reported in this study. No significant difference in outcome.
Abaturov et al. (2014)	Evaluate the efficiency of Lacidofil® in the	Open label, controlled, clinical trial 2 groups with anti-	45 children with active chronic H.	0.3 billion cfu/day	21 days	• In probiotic group, there was a more rapid regression of the intensity of the pain syndrome,

	treatment of children with *H. pylori* infection	helicobacterial therapy - Control group (n=20) - Probiotic group (n=25): 1 capsules of Lacidofil® TID	pylori-associated gastroduodenitis (10-16 years)			dyspeptic syndrome • In the probiotic group,there was a significant increase in sCD14, which is associated with tendentious decreased activity of NF-κB. • Significantly higher *H. pylori* eradication was achieved by "triple" anti-Helicobacter pylori therapy with supplemental use of probiotic Lacidofil® compared to control (96% vs. 70%, p≤0.03). There was no report of adverse events.
Infants and children studies of Oralis SB® powder, a combination of *L. helveticus* Rosell®-52 (R0052) (54 %), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%) and *S. cerevisiae* var *boulardii* (7%). Each sachet of Oralis SB® powder contains 1.25x10^9 cfu of bacteria, corresponding to 6.7x10^8 cfu of *L. helveticus* Rosell®-52 (R0052).						
References	**Objectives**	**Study Design**	**Subject**	***L. helveti-cus* Dose/day**	**Duration**	**Safety-Related results**
Jindal et al. (2011)	Explore the effect of oral probiotics on salivary *Streptococcus mutans* counts and thus to evaluate if oral probiotics are a preventive tool against the development of tooth decay.	Double blind, randomized, controlled study 3 groups (n=50/group): placebo group / Darolac (other brand name Oralis SB) (1.25x10^9 cfu) group/ Sporolac (Bacillus coagulans) group Once daily: to dissolve one sachet contents in 20 ml of water and to use as a mouth rinse for 1 minute before swallowing it. 14 days	150 healthy children (7-14 years)	0.675 billion cfu/day	14 days	A significant reduction in salivary count of *S. mutans* (p≤0.001) after 14 days in both probiotics groups compared to placebo group. No adverse events were reported.
Thakkar et al. (2013)	Assess oral probiotics as a preventive tool	Double blind, randomized, Controlled study	90 healthy children (13-15	0.67 billion cfu/day	14 days	Probiotic and chlorhexidine caused significant inhibition of dental plaque accumulation

	against the development of tooth decay and periodontal disease	3 groups (n=30/group): placebo group / Darolac (other brand name Oralis SB) (1.25x10^9 cfu) group / Chlorhexidine group Once daily: to dissolve the sachet contents in 10 ml of distilled water and to use as a mouth rinse for 1 minute before spitting it. 14 days	years)			compared to placebo mouthwash. ($p \leq 0.001$). Probiotic mouthwash was significantly more effective for inhibition of dental plaque accumulation after 14 days of intervention and 3 weeks after discontinuation of intervention, compared to Chlorhexidine. There was no report of adverse events.
Puranaik et al. (2014)	Investigate if the product Darolac (other brand name Oralis SB) was effective in reducing plaque and gingivitis	Double blind, Randomized, Controlled study 3 groups (n=30/group): placebo group / Darolac (other brand name Oralis SB) (1.25x10^9 cfu) group / Chlorhexidine group Twice daily: to dissolve the sachet contents in 20 ml of water and to use as a mouth rinse for 1 minute before expectorate it. 14 days	90 healthy children (15-16 years)	1.34 billion cfu/day	14 days	Probiotic and chlorhexidine mouth rinses were able to significantly inhibition of plaque accumulation and gingival scores after 14 days ($p \leq 0.05$). Probiotic mouth rinse is significantly more effective on gingival scores ($p \leq 0.01$). No side effect has been reported in this study. (An examiner was responsible for conducting the enquiry on adverse events).

6.4.1.4. Conclusions from Studies in Infants and Children

Clinical data obtained from the safety study on the three individual notified probiotic strains *L. helveticus* Rosell®-52, *B. longum* ssp. *infantis* Rosell®-33, and *B. bifidum* Rosell®-71 (Manzano et al. 2017), showed that the consumption of these three strains was well tolerated and safe for infants from 3 to 12 months of age. No serious adverse events were reported.

Regarding the studies with Probiokid®, a combination of *L. helveticus* Rosell®-52 (80%), *B. longum* ssp. *infantis* Rosell®-33 (10%), and *B. bifidum* Rosell®-71 (10%), the quality of the reporting of adverse events (AEs) varied significantly between the studies. The investigator-initiated studies tend to be short reports and did not always provide sufficient detailing of the study. The authors generally stated that “no adverse events were observed” even when a criterion such as vomiting was part of the inclusion. Thus, the authors may simply have been reporting that no unexpected AEs were reported or that no serious AEs were observed.

The paper by Pantovic (2013) was negligent in reporting AEs while the sponsored study from France (Cazzola et al. 2010b) detailed clearly the AEs in both arms of the study. In the French study there were 24 AEs reported in 20 children (9 in the placebo arm and 11 in the Probiokid® arm). None of the AEs was serious and only two AEs associated with digestion were considered by the investigators as possibly related to the study placebo. There were no AEs considered by the investigators to be related to the ingestion of Probiokid®. The main complaints were digestive problems (5 in placebo group; 1 in Probiokid® group) and varicella (2 in placebo group; 3 in Probiokid® arm). Dysuria and flu-like symptoms were reported equally in both the Probiokid® and placebo arms. All other AEs were single incidences of typical childhood infections such as otitis or injuries such as ankle sprains.

Moreover, Probiokid® is included in an ongoing program of pharmacovigilance for monitoring adverse events. Probiokid® has also been the subject of a Periodic Safety Update Report (PSUR), which outlines the safety profile of this product. The first PSUR covers the period from 01 January 2006 through 31 December. During that time, a total of 574,205,358 sachets of Probiokid® were sold globally. Only four non-serious adverse events were reported during this period (3 spontaneous reports and 1 health authority report – see Table 40). For the PSUR covering the period from 01 January 2013 through 31 December 2016, a total of 564 810 364 sachets of Probiokid® were sold no non-serious or serious adverse events have been reported.

Table 40. List of Reported Non-Serious Adverse Events – (PSUR: 01 January 2006 through 31 December 2012).

Case Number	Age & Sex	Confounding Factors	Complaint	Causality assessment
LAL100004 (Health authority)	13 month-old, unspecified gender	Concomitant vaccination with Twinrix® for Hepatis A & B	Urinary tract infection due to *E. coli*. Medically confirmed.	Deemed unlikely to be related.
LAL110005 (Spontaneous)	Not provided	Not reported	Vomiting, nausea and stomach ache, not medically confirmed	Possibly but insufficient information for full assessment.
LAL110010 (Spontaneous)	Not provided	Not reported	Vomiting after each dose of synbiotic, not medically confirmed	Possibly but insufficient information for full assessment.
LAL110029 (Spontaneous)	19 year old female	Medical history of sensory neuropathy of multineuris type with positive cryoglobulin and concomitant intake of Jasminelle as oral contraceptive	Throat irritation after 4 days followed 24 hours later by swollen lymph nodes in neck, muscle ache, extreme fatigue accompanied by bowel motility disorder. No fever. She continued to take the synbiotic and effect diminished. Not medically confirmed	Deemed unlike to be related.

In conclusion, taking account of the studies cited above, the lack of serious adverse events during clinical studies combined with pharmacovigilance monitoring demonstrate that the notified probiotics, both individually and in combination, are safe for use in children from one month of age to 7 years.

6.4.2. Studies in Adults

6.4.2.1. Studies of the Three Individual Notified Probiotic Strains

The research studies discussed below are summarized in Table 41 at the end of this section.

A double-blind, randomized, placebo-controlled study by Langkamp-Henken et al. (2015) is a 4-arm study designed to evaluate the effect on the proportion of healthy days (i.e., days without symptoms of cold or flu) of the three bacterial strains contained in Probiokid®: *B. longum* ssp. *infantis* Rosell®-33 (R0033), *L. helveticus* Rosell®-52 (R0052), and *B. bifidum* Rosell®-71 (R0071), versus a placebo. The probiotics were supplied as off-white powder packed in capsule containing $3x10^9$ cfu. The placebo was composed of magnesium and potato starch.

A total of 581 participants aged >18 years started the study protocol daily over a 4-day period. Initially, participants completed 7 days of pre-baseline questionnaires (week 0) and were then randomly assigned to receive an investigational supplement (*L. helveticus, B. bifidum* or *B. longum* ssp. *infantis*) or placebo. Participants completed daily questionnaires, consumed the study supplements, and were followed for the next 6 weeks. The complete study duration was 7 weeks.

The 581 students were randomly allocated into 4 groups:

- 145 healthy student*s* received one capsule of *B. longum* ssp. *infantis* Rosell®-33 ($3x10^9$ cfu)
- 142 healthy students received one capsule of *B. bifidum* Rosell®-71 (R0071) ($3x10^9$ cfu),
- 147 healthy students received on capsule of *L. helveticus* Rosell®-52 ($3x10^9$ cfu),
- 147 healthy students received one capsule of placebo

once daily, with a meal, for 6 weeks.

The study analysis and reporting of the results was focused on this one arm only. The unblinding of the study revealed that the arm which showed positive results was *B. bifidum* Rosell®-71 (R0071).

Over the course of the study, three participants withdrew:

- the first participant withdrew after taking *L. helveticus* Rosell®-52 for 7 days due to a loss of interest in participating;
- the second withdrew after 1 day on *B. longum* ssp. *infantis* Rosell®-33 due to gastrointestinal discomfort;
- the third withdrew after taking the placebo for 25 days due to abdominal pain.

Additionally, throughout the study, one participant complained of increased appetite (placebo) and another of a 'popping knee' while exercising (*L. helveticus* Rosell®-52). After approximately 2 weeks of supplementation, two participants discontinued the supplement (placebo and *B. longum* ssp. *infantis* Rosell®-33) due to diarrhea, but completed the remainder of the study-related activities.

There was no report of serious adverse events.

Table 41. Studies of Three Probiotic Strains: *B. longum* ssp. *infantis* Rosell®-33 (R0033), *L. helveticus* Rosell®-52 (R0052), and *B. bifidum* Rosell®-71 (R0071).

References	Objectives	Study Design	Subject	Strain Dose/Day	Duration	Safety-Related results
Langkamp-Henken et al. (2015)	Evaluate the effect on the proportion of healthy days (i.e. days without symptoms of cold or flu)	Double-blind, randomized, placebo-controlled study R0071:(n=142) 1 cap/day R0033: (n=147) 1 cap/day R0052: (n=145) 1 cap/day Placebo: (n=147)	581 healthy students	$3x10^9$ cfu/day	6 weeks	There was no report of serious adverse events. One participant withdrew after one day of R0033 because of abdominal pain. After 2 weeks, one participant on R0071 stopped treatment due to diarrhea. On placebo, one participant withdrew after 25 days because of abdominal pain, another stopped treatment after 2 weeks due to diarrhea. Significantly greater proportion of healthy days in *B. bifidum* group compared to placebo ($p \leq 0.05$)

*cfu: Colony Forming Units

6.4.2.2. Studies of *L. helveticus* Rosell®-52 (R0052) in Other Formulations

As cited previously, in addition to inclusion in the Probiokid® formulation, the strain *L. helveticus* Rosell®-52 (R0052) has also been extensively marketed for adults by Lallemand Health Solutions as a part of the four following blends:

- Probio'Stick®, a combination of *L. helveticus* Rosell®-52 (R0052) (89.4%) and *B. longum* ssp. *longum* R0175 (10.6%). Each sachet of ProbioStick® contains $3x10^9$ cfu, corresponding to $2.7x10^9$ cfu of *L. helveticus* Rosell®-52 (R0052).
- Lacidofil®, a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus* Rosell®-52 (R0052) (5%). Each capsule of Lacidofil® contains $2x10^9$ cfu, corresponding to 10^8 cfu of *L. helveticus* Rosell®-52 (R0052).
- Oralis SB® powder, a combination of *L. helveticus* Rosell®-52 (R0052) (54%), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%) and *S. cerevisiae* var *boulardii (7%).* Each sachet of Oralis SB® powder contains $1.25x10^9$ cfu of bacteria, corresponding to $6.7x10^8$ cfu of *L. helveticus* Rosell®-52 (R0052).

Each product containing *L. helveticus* Rosell®-52 has been studied for safety and efficacy in adults. The research studies discussed below are summarized in Table 42 at the end of this section.

- ***Clinical Studies of Probio'Stick®***

Probio'Stick® is a combination of *L. helveticus* Rosell®-52 (R0052) (89.4%) and *B. longum* ssp. *longum* R0175 (10.6%). Each sachet of ProbioStick® contains $3x10^9$ cfu, corresponding to $2.7x10^9$ cfu of *L. helveticus* Rosell®-52 (R0052).

Diop et al. (2008) showed statistically significant beneficial effects on GI symptoms in patients subjected to chronic stress while Messaoudi et al. (2011) demonstrated significant beneficial psychological effects in healthy humans. These 2 studies employed a daily dose of $3x10^9$ cfu of Probio'Stick®, corresponding to a dose of $2.7x10^9$ cfu of *L. helveticus Rosell®-52 (R0052)* per day. In both studies, it was reported that no adverse reactions were observed and the product was safe and well tolerated during the entire period of supplementation.

Another clinical study with Probio'Stick® demonstrated its beneficial effect in the reduction of signs and symptoms associated with IBS (Morales-Suarez et al. 2013). This study was a prospective observational multicenter study performed on 251 volunteer patients. Patients were administered Probio'Stick® 60 days. The symptoms did not always disappear, but improvement was observed in the quality of life. No adverse effects were reported during treatment.

- ***Clinical Studies of Lacidofil®***

Lacidofil®, containing 95% *L. rhamnosus* R0011 and 5% *L. helveticus Rosell®-52 (R0052)*, has been assessed in the clinical studies described below. Each capsule of Lacidofil® contains $2x10^9$ cfu, corresponding to 10^8 cfu of *L. helveticus* Rosell®-52

(R0052). Lacidofil® has proved to be efficient in the reduction of GI discomfort in 3 studies, Benes et al. (2006), Zvyagintzeva and Plutenko (2008), and Lee et al. (2014).

Benes et al. (2006) conducted an open-label study in 50 hospitalized adult patients with demonstrable chronic IBS. All patients exhibited two or more Manning criteria. The most frequently reported symptoms were feelings of abdominal pressure and bloating, flatulence and gas, rumbling or sloshing of intestinal contents. Many also suffered a high frequency of defecation. Prior to beginning the study, all subjects underwent a comprehensive examination including negative colonoscopy or irrigoscopy. All had negative bacteriological stool tests. Subjects were instructed to take 1 Lacidofil® capsule three times daily ($6x10^9$ cfu per day) for 4 months. The supplementation was well tolerated, with no side effects; all completed their four-month course of treatment with Lacidofil®. More than 80% of subjects reported a reduction in abdominal pressure and bloating and a reduction in the frequency of defecation with a favorable change in stool consistency. More than 60% of subjects also reported a marked reduction in flatulence and gas production and abdominal rumbling and sloshing. Upon completion of the study, 14 subjects continued the therapy of their own will. In 20 subjects, aggravation (or reappearance of abdominal discomfort) occurred within 4 months after conclusion of the study; of those, 15 resumed taking the therapy. Ten patients who completed the treatment reported no major deterioration in the first four months but only three patients continued to be symptom-free at four to eight months following completion of the study. The treatment was well tolerated, and none of the patients had any complaints or developed any side effects.

In an open-label study conducted by Zvyagintzeva and Plutenko (2008), researchers found that Lacidofil® supplementation (2 to $6x10^9$ cfu/day for 3 weeks) completely restored eubiosis in 85% of patients and improved the microbiotic composition in the remaining 15% of patients with dysbiosis related to IBS. After the treatment course, dyspeptic symptoms (abdominal pain, creatorrhea, steatorrhea, amylorrhea, and polyfaecalia) disappeared in 18 of 20 patients (90%), although abdominal distension was occasionally observed in three patients (15%). No adverse events were reported.

Lee et al. (2014) showed that Lacidofil® is effective in resolving bowel symptoms and improving quality of life in colorectal cancer survivors. A randomized, double-blind, placebo-controlled study was performed on patients > 20 years of age diagnosed with stage 2 or 3 colorectal cancer who had been performing well or who completed treatments between 6 weeks and 2 years prior. Sixty-six participants were randomly allocated to receive Lacidofil® or a placebo for 12 weeks. All participants completed anthropometric measurements and completed questionnaires about health-related lifestyles, underlying diseases, quality of life, and bowel symptoms (bloating, abdominal pain, excessive gas, diarrhea, etc.). For the measurement of the bowel symptoms, the diagnosis of IBS (ROME II criteria) was used.

Upon enrollment in the study, there was no significant difference between participants who suffered irritable bowel symptoms (65.6% of the placebo group vs. 67.9% of the probiotics group). At the end of the treatment, the proportion of participants exhibiting these symptoms decreased to 45.7% in the probiotics group while 62.5% of placebo group still suffered irritable bowel symptoms ($p \leq 0.03$). The proportion of participants exhibiting bowel symptoms were significantly different between two groups ($p \leq 0.05$). Indeed, twelve weeks of supplementation with probiotics significantly decreased the proportion of patients suffering from irritable bowel symptoms and improved colorectal cancer-related quality of life (FACT-C), fatigue-related quality of life (FACT-F), and mental health scores (PHQ-9). The differences in the proportion of patients with bowel symptoms, the mean changes of colorectal cancer-related quality of life scores, and functional well-being scores were significantly greater in the probiotic group compared with the placebo group. No significant side effects or harmful events were reported during the treatment in either group.

AAD is a common complication of antibiotic use, which can occur after exposure to antibiotics as a result of disrupting the intestinal microbiota. One of the roles of the intestinal microbiota is to act as a protective barrier that resists the colonization of intestinal pathogens. Without this protective barrier, patients are susceptible to infection by opportunistic pathogens. Lacidofil® has been shown to fight against AAD and pathogens associated with it.

In a study by Song et al. (2010), 214 adult patients being treated with antibiotics for respiratory tract infections were randomized to receive Lacidofil® ($4x10^9$ cfu/day) or a placebo for 14 days. The number of adults who developed AAD in this study was low. AAD developed in 4 (3.9%) of 103 patients in the Lacidofil® group and in 8 (7.2%) of 111 patients in the placebo group ($p \leq 0.44$). This was not considered a significant difference between groups. This result may be attributed to the short-term follow-up period (14 days) because AAD can occur up to 2 months after the end of antibiotic treatment. But the Lacidofil® group did experience less change in bowel frequency and consistency (50/103, 48.5%) than the placebo group (35/111, 31.5%) ($p \leq 0.01$). In this study, mild abdominal pain was reported in three patients and a skin eruption in one patient receiving Lacidofil®, but the authors concluded that these adverse events could not be attributed to the use of Lacidofil®.

Evans et al. (2015) investigated the effect of Lacidofil® Strong supplementation on AAD in healthy adults. Lacidofil® Strong contains the same bacteria as Lacidofil®, *L. rhamnosus* R0011 and *L. helveticus* R0052, in the same ratio (95:5) but at a higher concentration of $4x10^9$ cfu/capsule instead of $2x10^9$ cfu/capsule, and therefore $2x10^8$ cfu of *L. helveticus* R0052. The primary goal of this study was to determine the effect of probiotic supplementation with antibiotic use on consistency and frequency of bowel movements. Secondary outcome measures included the proportion of participants reporting diarrhea-like defecations (DLD), GI symptoms, and adverse events. The study

was a randomized, double-blind, placebo-controlled parallel trial conducted over 10 weeks with five distinct periods: run-in from day -7 to baseline, antibiotic + treatment or placebo from day 1 to 7, treatment or placebo only from day 8 to 14, no treatment or placebo from day 15 to 21, and follow up from day 22 to 63.

A total of 160 healthy adults aged 18-50 years were randomly allocated to either treatment (n=80) or placebo (n=80). Participants were instructed to take one capsule of amoxicillin-clavulanic acid before breakfast and another before dinner, while one capsule containing $4x10^9$ cfu of Lacidofil® or placebo was taken with each of those meals. The total dose per day was $8x10^9$ cfu.

The primary outcome measure showed a significant increase in both groups in Bristol Stool Scale – BSS (consistency of bowel movement) scores during the antibiotic treatment period compared to the run-in period in both groups (probiotic $p \leq 0.001$; placebo $p \leq 0.0001$). There were no significant differences in the weekly mean of daily BSS values between the probiotic and placebo groups at any time point during the study. Additionally, there was a significant increase in the frequency of bowel movements during the amoxicillin-clavulanic acid treatment period compared to the run-in period in both the probiotic ($p \leq 0.036$) and placebo ($p \leq 0.038$) groups. This increase returned to baseline values by the follow-up period, but there were no significant differences in bowel movement frequency between treatments.

There were no significant differences in GI symptoms between groups in constipation, abdominal pain, and indigestion. However, the proportion of participants reporting diarrhea-like defecations (DLD) was lower in the probiotic group than in the placebo group. The duration of DLD events with was significantly reduced with Lacidofil® supplementation compared to placebo ($p \leq 0.037$; effect size=0.52).

A total of 139 adverse events was reported during this trial, with 52% of participants experiencing at least one adverse event. Of these events, 29 (9 probiotic, and 20 placebo) were categorized as 'possibly related' to the investigational product. Two study participants from the placebo group withdrew from the study because of adverse events. Biometric, vital, and hematological parameters were normal throughout study

In addition, another study was performed on patients with only acute diarrhea. Simadibrata et al. (2012) conducted a clinical trial to assess the effect of Lacidofil® on acute diarrhea in adult patients, including diarrhea duration, stool frequency and consistency, and abdominal complaints and other complications.

In this randomized, double-blind, placebo-controlled clinical trial, 90 adult patients aged 21-49 years with acute diarrhea and without severe complications were randomly allocated to either the Lacidofil® (n=45) or placebo group (n=45). The Lacidofil® group received oral rehydration salt (Oralit®) and two capsules three times per day of Lacidofil® for 7 days. Each Lacidofil® capsule contained 1.9×10^9 cfu of *L. rhamnosus* R0011 and 10^8 cfu of *L. helveticus* R0052. The total dose per day was $12x10^9$ cfu. The

placebo group received oral rehydration salt and placebo capsule at equal dose of 3 × 2 capsules for 7 days.

The clinical presentation of illness at baseline and final, including symptoms of diarrhea, abdominal complaints, and other complications were recorded, and whole blood and stool samples were collected. Stool consistency and severity of diarrhea, as well as other symptoms (abdominal pain, nausea, vomiting, bloating, tenesmus, headache, and daily activity disturbance) were monitored throughout the study period.

The duration of diarrhea in the Lacidofil® group was significantly less than in the placebo group ($p \leq 0.018$), which demonstrated significantly faster recovery from diarrhea. Frequency of stools, stool consistency, abdominal pain, nausea, vomiting, bloating, headache, fever, and tenesmus were improved in the Lacidofil® group than in the placebo group. No adverse events were reported in either group.

Patients being treated with antibiotics for *Helicobacter pylori* infections may also benefit from Lacidofil®. Ziemniak (2006) designed a controlled study to evaluate the efficacy of usual treatments against *H. pylori* infection and the resistance of *H. pylori* strains to antibiotics. The effects of a combined treatment with antibiotics and Lacidofil® on *H. pylori* eradication were also studied in order to suggest improvements of the recommended therapies. A group of 641 patients with endoscopically-detected gastric or duodenal ulcers, chronic gastritis, and confirmed *H. pylori* infection were divided according to anti-*H. pylori* scheme:

- group IA: amoxicillin + clarithromycin + proton pump inhibitor (PPI), twice daily for 10 days
- group IB: tetracycline + tinidazole + bismuth salts + PPI, 4 times per day for 10 days
- group II: according to the antibiogram
- group III: patients from group I still having *H. pylori* infection at 6 weeks after treatment, and other patients treated unsuccessfully in the past, treated according to the antibiogram
- group IV: amoxicillin + clarithromycin + PPI + Lacidofil®, twice daily for 10 days ($8x10^9$ cfu/day).

H. pylori infections were detected by ^{13}C-urease breath test (UBT), and by microbiological tests for groups II and III.

The eradication rate was higher in patients in group II treated according to the antibiogram (94.3%) and in patients treated with antibiotics and supplemented with Lacidofil® (94.3%) when compared to other patients ($P \leq 0.05$). The improved effectiveness of the amoxicillin + clarithromycin + PPI triple treatment observed in patients supplemented with Lacidofil® (group IV vs. group IA) may be due to the direct antagonistic action of *Lactobacillus* strains on *H. pylori*. The author concluded that

Lacidofil® appears as a promising treatment to reduce the use of antibiotics in the treatment of *H. pylori* infections. No adverse events were reported.

Similar results were found in a clinical study by Bielanski et al. (2002) which reported an *H. pylori* eradication rate significantly higher in a group with Lacidofil® supplementation to therapy compared to controls ($p \leq 0.05$). Out of 99 patients who completed conventional anti-*H. pylori* therapy, 72% were successfully eradicated, while among 51 patients whose therapy was supplemented with Lacidofil®, the *H. pylori* eradication rate significantly increased up to 92%. No moderate or severe side effects were reported in the probiotic group; in the control group, one patient with severe diarrhea left study early.

Babak (2007) randomized 35 adults with uncomplicated duodenal ulcers associated with *H. Pylori*. Twenty patients received Lacidofil® (1.2×10^{10} cfu/day for 20 days) in addition to the standard triple antibiotic therapy and 15 patients received only the standard triple therapy. Eradication of *H. Pylori* was seen in 18 of 20 patients receiving Lacidofil® and in 13 of 15 patients in the control group. Dyspeptic symptoms were corrected more quickly in the treatment group (6.0 ± 0.6 days) compared to the control group (10.0 ± 1.1 days) ($p \leq 0.01$). Assessment of the quantitative content of different intestinal microbiota types showed that treatment with Lacidofil® led to an increase of the number of bifidobacteria and lactobacilli and disappearance of opportunistic microorganisms in comparison with the control group, as well as an increased number of patients where restoration of the intestinal microbial pattern was observed. No significant side effects were observed during the study, and the patients reported good tolerance of the probiotic preparation.

Vdovychenko et al. (2008) reported on a clinical trial comparing treatment efficiency of patients with duodenal peptic ulcer by conventional triple treatment regime and the same treatment with probiotic administration. Forty-nine patients aged between 41 and 47 years with duodenal peptic ulcer were selected and divided into 2 groups. The placebo group (n=24) was treated according to the conventional triple scheme of Omeprazole, Amoxicillin, and Clarithromycin for 7 days. Patients in the probiotic group (n=25) were treated with the same conventional treatment and with Lacidofil® at a dose of 2 capsules twice daily for 10 days. The total dose per day was 8×10^9 cfu.

Patients in the probiotic group had significantly quicker regression of clinical signs of disease (pain after 2-3 days of treatment, dyspeptic syndrome on the 4th day of treatment) while for those in the placebo group pain syndrome was removed only on the 3rd - 4th day and dyspeptic disorders the 5th day of treatment. Addition of probiotics also significantly reduced the frequency of side effects of antihelicobacter therapy action in patients, e.g., frequency of detection of duodenitis in patients of the placebo group almost did not change (95.8 vs 91.7% before treatment), whereas it was lower in patients of the probiotic group after treatment (76 vs 96% respectively). The results also showed a statistically significant difference in *H. pylori* eradication between the probiotic

group and control group (96 vs 75 % respectively). Healing of the erosive-ulcerative effect was significantly faster in patients in the probiotic group compared to patients in the placebo group (88.0 vs 70.8% respectively). Only one patient in the probiotic group reported side effects (eructation and dry mouth), versus six in the control group.

Two studies (Chayka et al. 2006 and Liskovich et al. 2010) have demonstrated that Lacidofil® may aid in the prevention of disbacteriosis in pregnant woman recently confined after Caesarean operation.

In Chayka et al. (2006), 103 pregnant women who planned delivery by Caesarean section, and under antibiotic treatment, were allocated to one of the following groups:

Group I (n=38) received one Lacidofil® capsule twice a day ($4x10^9$ cfu/day) for 5-6 days before and for 10 days after the operation.

Group II (n=35) received only one Lacidofil® capsule twice a day for 10 days after the operation.

Group III (n=30) did not receive probiotics.

After 10 days of treatment, the presence of symptoms of dysbacteriosis (meteorism, dyspepsia, and abdominal discomfort) was observed only in patients from Groups II and III, and in Group III significantly more often than in Group II. In addition, the results on the examination of the discharge from maternal passage showed a significant reduction of pathogen microorganisms (*E.coli, S. aureus*, and Candida) in Group I compared with Groups II and III ($p \leq 0.05$). Results on the microbiological examination of the amniotic liquid showed a significant reduction of pathogens in Group I compared with Groups II and III ($p \leq 0.05$). Furthermore, no *E. coli* and no *S. aureus* was found in Group I. There was a statistically significant reduction in *E. coli* and Candida from the stomachs of the newborns in Groups I and II compared to Group III. No adverse events were reported among either the women or their newborns.

Liskovich et al. (2010) randomized 96 women receiving prophylactic antibiotic therapy (Cefotaxime for prevention of pyroseptic complications) after Cesarean section delivery into a treatment group (n=56) that received $6x10^9$ cfu/day of Lacidofil® for 7 days and a control group (n=40) that received only the antibiotic treatment.

In patients receiving Lacidofil® supplementation, 89.3% were considered eubiotic following therapy, while none of the patients of the control group were eubiotic. AAD did not develop in any of the patients receiving Lacidofil®, but AAD was recorded in 10% of patients from the control group ($p \leq 0.05$). There was no report of adverse events

A clinical study (Kocian 1994) was conducted to highlight the effect of Lacidofil® in adult patients suffering from lactose intolerance. Patients enrolled in this study were experiencing dyspepsia caused by enteric dysbiosis. The 21 patients recorded their tolerance for the studied food while fasting in the morning before treatment and after 14 days of regular administration of one capsule daily of Lacidofil® ($2x10^9$ cfu) after

breakfast. Before the treatment, 19 patients tolerated only 20-30 ml milk and 40-70g cheese. After 14 days of treatment, tolerance increased to 40-50 ml milk and 70-80 g cheese. In addition, the number of stools decreased significantly ($p \leq 0.01$) after Lacidofil® treatment, and the average improvement in stool consistency scores was also significant. The total intolerance score (meteorism, flatulence, stomach pain, and diarrhea) also decreased statistically significantly. There were no reports of adverse events, and liver and kidney parameters and blood counts did not change. The preparation was well tolerated.

In an open-label clinical study aimed at assessing the ability of *L. rhamnosus* R0011 and *L. helveticus* R0052 to survive during passage through the human digestive tract and to influence the equilibrium of the microbiota (Firmesse et al. 2008), 14 apparently healthy adults were given 4 capsules of Lacidofil® daily for 12 days after a 3-week exclusion period of fermented products. The population of *L. rhamnosus* R0011 found in the faces after the consumption period was high, with a mean value of 7.1 $\log^{10}$ cfu equivalents/g of stool, while *L. helveticus* R0052 was found in only one patient at a maximal level of 6.1 $\log^{10}$ cfu equivalents/g of stool. The dominant microbiota composed of *Clostridium coccoides*, *Bifidobacterium* sp., *Bacteroides* sp., and *Clostridium leptum* groups, seems to have not been influenced by the consumption of probiotic bacteria. No changes were seen in the overall microbiota profile of participants which was not surprising given the study group was composed of healthy individuals with presumably a balanced gut microbiome.

Dahl et al. (2016) performed a randomized trial to determine the effects of calcium carbonate and calcium phosphate supplementation on faucal *Lactobacillus* spp., with and without a probiotic supplement, in healthy adults. Study 1 and Study 2 were both randomized double-blind crossover trials. In study 1, participants (n=15) received 2 capsules/day of calcium carbonate (Ca1) and calcium phosphate (Ca2) each for 2-week periods, with 2-week baseline and washout periods. In study 2, participants (n=17) received 2 capsules/day of *L. helveticus* Rosell®-52 (R0052) and *L. rhamnosus* R0011 alone, the probiotic with 2 capsules/day of Ca1, and probiotic with 2 capsules/day of Ca2 each for 2-week periods with 2-week baseline and washout periods. In both studies, stools were collected during the baseline, intervention and washout periods for *Lactobacillus* spp. quantification and qPCR analyses. Participants completed daily questionnaires of stool frequency and compliance.

In Study 1, neither calcium supplement influenced counts of resident *Lactobacillus* spp., genome equivalents of lactic acid bacteria, or stool frequency. However, in Study 2, fecal *Lactobacillus* spp. counts were significantly enhanced from baseline when the probiotic was administered with Ca2 ($p \leq 0.02$), but not with Ca1 or with the probiotic alone. No adverse events were reported.

In an open-label study (Wojoik et al. 1996; unpublished), effectiveness and tolerance of Lacidofil® were evaluated in 28 adult patients who were suffering from chronic

constipation, undergoing long term postoperative antibiotic administration, or had short bowel syndrome due to extensive resection of the small intestine and right-side hemicolectomy. All patients were given Lacidofil® in doses of 2 capsules 3 times daily with meals for 14-26 days. In patients with chronic constipation, significant improvement was recorded with 64% of the patients reporting daily stool passage after 14 days of treatment ($p \leq 0.05$). Lacidofil® had no effect on the frequency of stool passage for the short bowel syndrome patients. Improvement in stool consistency was noted for chronic constipation patients after 14 days of treatment with Lacidofil®. The authors concluded that Lacidofil® was effective in restoring normal stool frequency and consistency in patients suffering from chronic constipation, though this was not the case for the patients with short bowel syndrome. The preparation was well tolerated by all patients.

- ***Clinical Studies on ORALIS SB® powder®* (commercialized under the Brand name Darolac® by Aristo Pharmaceutical)**

Oralis SB® powder® is a combination of *L. helveticus* Rosell®-52 (R0052) (54 %), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%) and *S. cerevisiae* var *boulardii (7%).* Each sachet of Oralis SB® powder contains 1.25×10^9 cfu of microorganisms, corresponding to 6.7×10^8 cfu of *L. helveticus* Rosell®-52 (R0052).

In a randomized single-blind study, Jothika et al. (2015) analyzed the short-term effectiveness of probiotics, chlorhexidine, and fluoride mouth rinse on plaque *S. mutans* level. Fifty-two healthy adult patients aged 18-25 years with good dental health were randomly allocated in four groups:

- Group 1 (n=13): placebo group: each subject used 10 ml of distilled water
- Group 2 (n=13): chlorhexidine group: each subject used 10 ml of 0.2% chlorhexidine mouthwash
- Group 3 (n=13): fluoride group: each subject used 10 ml of 500 ppm F/400 ml sodium fluoride mouthwash
- Group 4 (n=13): probiotic group: each subject used 10 ml of Oralis SB®.

All volunteers were instructed to rinse their mouth twice daily after brushing with a non-fluoridated toothpaste for 30 days. Buccal plaque samples were collected 8 minutes after the sucrose challenge at baseline (24 hours) and after 7, 14, and 30 days.

The comparison of Group 1 vs. Groups 2, 3, and 4 showed a statistically significant difference in the mean colony count of *S. mutans* ($p \leq 0.05$). However, there was no statistically significant difference among Groups 2, 3, and 4 ($p > 0.05$). There was no report of adverse events.

Table 42. Studies in Adults with *L. helveticus* R0052 Used in Other Formulations.

Adult studies of Probio'Sstick: a combination of *L. helveticus Rosell®-52 (R0052)* (89.4%) and *B. longum ssp. longum* R0175 (10.6%). Each sachet of Probio'Stick® contains $3x10^9$ cfu, corresponding to $2.7x10^9$ cfu of *L. helveticus Rosell®-52 (R0052)*.						
References	**Objectives**	**Study Design**	**Subject**	***L. helveticus* Dose /Day**	**Duration**	**Safety-Related results**
Diop et al. (2008)	Investigated the effects of Probio'Stick® on stress-induced gastrointestinal and psychological symptoms, on volunteers with symptoms of stress	Double-blind, placebo-controlled, randomized study. 1 group received Probio'Stick® ($3x10^9$ cfu/sachet) (n=37) and second group received placebo (n=38). Ingested once/day for 3 weeks	75 volunteers (18-60 years)	2.7 billion cfu/day	3 weeks	Significant improvement of stress-induced gastrointestinal symptoms of abdominal pain ($p \leq 0.004$) and nausea/vomiting ($p \leq 0.009$), in the Probio'Stick® group compared to placebo. Probio'Stick® consumption also tended to decrease flatulence and gas production. No adverse reactions were reported during the study. The product was safe and well tolerated during the entire period of supplementation.
Messaoudi et al. (2011)	Investigated the possible effects of Probio'Stick® on anxiety, depression, stress and coping strategies in healthy human volunteers	Double-blind, controlled, randomized, parallel study. Each participant then received thirty sticks of the Probio'Stick® ($3x10^9$ cfu/sachet) or placebo for thirty days	66 healthy subjects	2.7 billion cfu/day	3 weeks	Consumption of Probio'Stick® mitigated psychological distress in three tests and decreased urinary cortisol values ($p \leq 0.05$), without displaying any adverse event.
Morales-Suarez et al. (2013)	Demonstrate the beneficial effect in the reduction of signs and symptoms of IBS	Longitudinal, prospective observational, multicenter study. Patients were administered Probio'Stick® ($3x10^9$ cfu per sachet) for 60 days. Each patient was instructed to swallow the contents of one sachet every day for 60 days.	251 volunteers with clinical symptoms of IBS	2.7 billion cfu/day	60 days	Decrease in the various clinical manifestations of patients at the end of the 60 days of Probiotic treatment (distension, abdominal pain, flatulence, constipation, feeling of incomplete evacuation). No adverse effects were reported during treatment

Adult studies of Lacidofil: a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus Rosell®-52 (R0052)* (5%). Each capsule of Lacidofil® contains $2x10^9$ cfu, corresponding to 10^8 cfu of *L. helveticus Rosell®-52 (R0052)*						
References	**Objectives**	**Study Design**	**Subject**	***L. helveticus* Dose /Day**	**Duration**	**Safety-Related results**
Kocian (1994)	Efficacy and safety of lactobacilli in the treatment of dyspepsia caused by dysbacteriosis of gastrointestinal tract	Uncontrolled. 1 Lacidofil® capsule/day for 2 or 3 weeks; oral	30 adults	0.1 billion cfu/day	14 or 21 days	Changes in self-evaluation of pain, pressure, bloating, flatulence, appetite, stool frequency. Those with lactose intolerance experienced improved tolerance. The most rapid relief of symptoms was recorded in the subgroup of patients suffering from AAD in which the condition was normalized within 3- 4 days of treatment. There was no report of adverse events; liver and kidney parameters and blood counts did not change. Preparation was well tolerated.
Wojoik et al. (1996; unpublished)	Efficacy and tolerance of Lacidofil® in restoration of intestinal microflora, normalization of stool frequency/ consistency	Uncontrolled. 2 Lacidofil® capsules TID	28 adults suffering from GI and bowel issues	0.6 billion cfu/day	14-26 days	Change in frequency & consistency of stools, rectal microflora. Lacidofil® was highly effective in alleviating postprandial meteorism in patients with chronic constipation or with constipation related to gall bladder calculosis as well as in patients with short bowel syndrome. ($p \leq 0.05$) The preparation was tolerated by all patients
Bielanski et al. (2002)	Efficacy of standard triple anti - *H. pylori* therapy supplemented with Lacidofil	Active controlled 1 Lacidofil® capsule daily Orally with conventional anti-Hp therapy Group A: (n=99) standard anti Hp-therapy for 10 days Group B : (n=51) standard anti Hp-therapy for 10 days + Lacidofil® ($4x10^9$ cfu) tid, 20 days	150 adults with upper digestive tract symptoms	0.6 billion cfu/day	20 days	Hp eradication rate was significantly higher in probiotic group compared to controls ($p \leq 0.05$). No moderate or severe side effects in probiotic group, versus control group had one patient with severe diarrhea who left study early, and more taste discomfort.

Benes et al. 2006	Efficacy in reduction of complaints associated with IBS	Uncontrolled. 1 Lacidofil® capsule TID	50 adults (30-83 years) with chronic IBS	0.3 billion cfu/day	4 months	After supplementation, the frequency of defecation was significantly reduced and a favorable change in stool consistency was noted in 84% of patients; a reduction of abdominal pressure and bloating in 82% of patients; an improvement of abdominal and/or colic pain was also observed in 14 and 5 patients, respectively. There was a marked reduction in flatulence and gas production, and abdominal rumbling and sloshing. The treatment was well tolerated, and none of the patients had any complaints or developed any side effects.
Chayka et al. (2006)	Ability to prevent intestine dysbacteriosis in pregnant women recently confined with surgical delivery	Placebo controlled 3 groups under antibiotics - Group I (n=38): 1 Lacidofil® capsule BID before the operation for 5-6 days and also after the operation for 10 days - Group II (n=35): 1 Lacidofil® capsule BID after the operation for10 days - Group III (n=30): placebo	103 pregnant women who planned delivery by caesarian section. (21-32 years)	0.2 billion cfu/day	10 or 15-16 days	Introduction of Lacidofil® to preventive antibiotics helped avoid the negative effects of antibacterial preparations used for intraoperative and postoperative prophylaxis, such as dysbacteriosis ($p \leq 0.05$). There was no report of adverse events
Ziemniak et al. (2006)	Efficacy of a combined treatment with antibiotics and Lacidofil® on *H. pylori* eradication	Active-controlled 2 groups with anti-helicobacterial therapy - Control group (n=192) - Probiotic group (n=53): 2 Lacidofil® capsules BID, for 20 days	245 patients with gastric or duodenal ulcers and chronic gastritis with H. pylori infection (18-81 years)	0.4 billion cfu/day	20 days	Eradication higher in treatment group with probiotic, rather than treatment alone ($p \leq 0.04$). No adverse events were reported.

Babak et al. (2007)	Efficiency and safety of the standard antihelicobacterial regimen in combination with Lacidofil® in patients with duodenal peptic ulcers associated with *H. pylori*	Active controlled. 2 groups with anti-helicobacterial therapy (two antibiotics and a proton-pump inhibitor) - Control group (n=15) - Probiotic group (n=20): 2 Lacidofil® capsules TID, starting at the first day of anti-helicobacterial therapy, for 20 days	35 adults with uncomplicated duodenal ulcer associated with *H. pylori* (18-70 years)	0.6 billion cfu/day	20 days	Positive improvements to clinical symptoms occurred faster in Lacidofil® group compared to controls, especially for dyspeptic syndrome ($p \leq 0.01$).No significant side effects observed during the study, all the patients had good tolerance of the probiotic.
Firmess et al. (2008)	Assess the ability of R0011 and R0052 to survive during the passage through the human digestive tract	Open uncontrolled 3-week exclusion period of fermented products. 12-day consumption period (4 Lacidofil® capsules daily, $2x10^9$ R0011 and 10^8 R0052), 12-day wash-out period	14 healthy volunteers (18-50 years)	0.4 billion cfu/day	7 weeks (12 days of "treatment")	This study demonstrated that *L. rhamnosus* R11 provided as a food supplement could survive during passage through the human intestinal tract. No alteration was observed in microbiota of healthy subjects, suggesting the safety of this food supplement containing these 2 probiotic strains.
Vdovychenko et al. (2008)	Compare the treatment efficiency of patients with duodenal peptic ulcer (DDPU) by conventional triple treatment regime and quadruple therapy with probiotic administration	Active controlled 2 groups with anti-helicobacterial therapy - Control group (n=24) - Probiotic group (n=25): 2 Lacidofil® capsules BID, for 10 days	49 patients with duodenal peptic ulcers	0.4 billion cfu/day	10 days	Erosive ulcerative defects were healed faster in patients of the Group II. Addition of probiotics to the treatment regime facilitated a statistically significant reduction of frequency of side effects. Only one patient in probiotic group reported side effects (eructation and dry mouth), versus six in the control group. No adverse events were reported.

Zvyagintseva and Plutenko 2008	Study the effect of probiotic Lacidofil® on the clinical course of intestinal dysbacteriosis and degree of dysbiotic manifestations	Lacidofil® simultaneously with the basic therapy for IBS. Probiotic groups: Lacidofil® ($2x10^9$ cfu) For patient with 2nd degree dysbiosis (n=15): 3 capsules /day for 2 weeks, then 1 capsules /day for 1 week For patients with 3rd degree dysbiosis (n=5): 3 capsules /day for 2 weeks, then 2 capsules/day for 1 week, then 1 capsule/day for 1 week.	20 adults (25- 65 years) with IBS and medium severity diarrhea	Between 0.1 billion cfu/day and 0.3 billion cfu/day	3 or 4 weeks	After the treatment course by Lacidofil® probiotic, dyspeptic symptoms disappeared in 90% of patients. There was a significant ($p \leq 0.05$) positive influence on the qualitative and quantitative composition of colonic microflora. The authors did not report any adverse event.
Liskovich et al. (2009)	Efficacy of Lacidofil® for prevention of AAD during the postpartum period in women after cesarean operation	Controlled 2 groups under antibiotics Main Group (n=56): 1 Lacidofil® capsule TID for 7 days Control group (n=40): placebo	96 pregnant women who planned delivery by caesarian section. (21-33 years)	0.3 billion cfu/day	7 days	Manifestations of dysbiosis were stopped with the intake of probiotic after completion of the antibiotics-therapy course. No incidence of AAD was recorded in the main group of patients receiving Lacidofil® ($p \leq 0.05$), which was significantly less than control group. There was no report of adverse events.
Song et al. (2010)	Efficacy of Lacidofil®, for the prevention of AAD in adults	Randomized, placebo-controlled, double-blind trial 1 Lacidofil® capsule BID Treatment group (n=111): Lacidofil® cap Control group (n=103): placebo drug	214adults on antibiotic therapy for respiratory tract infection	0.2 billion cfu/day	14 days	Lacidofil® did not reduce the rate of occurrence of AAD in adult patients with respiratory tract infection who have taken antibiotics. The probiotics group maintained their bowel habits to a greater extent than the placebo group ($p \leq 0.01$). Mild abdominal pain was noted in 3 patients and a skin eruption in one patient receiving Lacidofil®, but these adverse events could not be attributed to the use of Lacidofil®

Simadibrata et al. (2012)	Efficacy of Lacidofil® in acute diarrhea caused by pathogens	Prospective, double blind, Randomized, placebo Controlled clinical trial. 2 groups: placebo group (n=38) Lacidofil® ($2x10^9$ cfu) group (n=38) 2 Lacidofil® capsules TID Both groups also received oral rehydration salt.	76 patients with acute diarrhea (21-49 years)	0.6 billion cfu/day	7 days	Faster recovery from diarrhea in probiotic group compared to placebo ($p \leq 0.018$) There was a greater improvement on clinical outcomes (frequency of stools, stool consistency, abdominal pain, nausea, vomiting, bloating, headache, fever and tenesmus). There were no adverse events in either group.
Lee et al. 2014	Evaluate the effect of Lacidofil® to resolve bowel symptoms and to improve quality of life in colorectal cancer (CRC) survivor	A randomized, placebo-controlled, double blind study. 2 groups: placebo group (n=32) / Lacidofil® ($2x10^9$ cfu) group (n=28) 1 Lacidofil® capsule BID for 12 weeks	66 colorectal cancer survivors suffering from IBS symptoms (aged>20 years)	0.2 billion cfu/day	12 weeks	<u>After the treatment:</u> • Decreased the proportion of patients suffering from IBS (from 67.9% to 45.7% in the Lacidofil® group vs. from 65.6% to 62.5% in the placebo group, $p \leq 0.03$). • Decrease in the proportion of patients with bowel symptoms significantly greater in probiotic group ($p \leq 0.05$). • Significantly improved colorectal cancer-related life quality (FACT-C) ($p \leq 0.04$), fatigue-related quality of life (FACT-F) ($p \leq 0.02$), and mental health scores (PHQ-9) ($p \leq 0.01$) over the 12 week period. No significant side effects or harmful events were reported during the treatment in either group.

Evans et al. (2015)	Efficacy of probiotic Lacidofil® STRONG, for the prevention of AAD in adults	Randomized, double-blind, placebo-controlled parallel study. 2 groups: placebo group (n=80) / Lacidofil® STRONG ($4x10^9$ cfu) group (n=80) 1 Lacidofil® capsule BID for 14 days. (Total dose /day: $8x10^9$ cfu) Each group given antibiotics for first week along with placebo or probiotic treatment	160 healthy subject (18-50 years)	0.4 billion cfu/day	10 weeks (2 weeks of treatment)	No significant difference on effect on bowel movements between treatment groups, but diarrhea-like defecation events were significantly shorter in duration in probiotic group ($p \leq 0.037$). A total of 139 adverse events were reported during this trial, with 52% of participants experiencing at least one adverse event. Of these events, twenty-nine (nine probiotic, and twenty placebo) were categorized as 'possibly related' to the investigational product, and were primarily gastrointestinal and infectious disorders. Two participants from the placebo group withdrew from the study due to adverse events: one withdrew during the antibiotic plus placebo period, the other reported mild to moderate GI symptoms related to bloating and gas experienced during the placebo-only period. Biometric, vital, and hematological parameters were normal throughout study.
Dahl et al. (2016)	Determine the effects of calcium carbonate and calcium phosphate supplementation on fecal *Lactobacillus* spp., with and without a probiotic supplement, in healthy adults	Randomized, double-blind crossover study. 1 at 2 Lacidofil® capsules/d of 250 mg (each) elemental calcium as calcium carbonate (Ca1) and calcium phosphate (Ca2) Study 2: 2 capsules/d of Lacidofil® alone, the probiotic with 2 capsules/d of Ca1, and probiotic with 2 capsules/d of Ca2.	Study 1: 15 healthy adults Study 2 17 healthy adults	0.2 billion cfu/day	2 weeks	Study 1: neither calcium supplement influenced viable counts of resident *Lactobacillus* spp., genome equivalents of lactic acid bacteria or stool frequency. Study 2: fecal *Lactobacillus* spp. counts were significantly enhanced from baseline when the probiotic was administered with Ca2 ($P \leq 0.02$), but not with Ca1 or with the probiotic alone. Detection of L. helveticus R0052 and L. rhamnosus R0011 was significantly increased with all treatments, but did not differ among treatments. No adverse events were reported.

Adult studies of Oralis SB® powder (other brand name DAROLAC): a combination of *L. helveticus Rosell®-52 (R0052)* (54 %), *L. rhamnosus* R0011 (29%), *B. longum ssp. longum* R0175 (10%) and *S. cerevisiae var boulardii (7%).* Each sachet of Oralis SB® powder contains $1.25x10^9$ cfu of bacteria, corresponding to $6.7x10^8$ cfu of *L. helveticus Rosell®-52 (R0052)*						
References	**Objectives**	**Study Design**	**Subject**	***L. helvet-icus* Dose/Day**	**Duration**	**Safety-Related results**
Jothika et al. (2015)	Analyze the effectiveness of probiotics on plaque *S. mutans* level	Single-blind, Randomized, Controlled study. 4 groups (n=13 / group): placebo group / Chlorhexidine group / Fluoride group/ Darolac (other brand name Oralis SB) group Twice daily: to dissolve the sachet contents in 10 ml of water and to use as a mouth rinse for 1 minute. 30 days	52 healthy adults (18-25 years)	1.34 billion cfu/day	30 days	Chlorhexidine, sodium fluoride and probiotic products have statistically similar and equivalent antimicrobial effects on plaque *S. mutans* level, all performed better than placebo ($p \leq 0.05$). No adverse events were reported.

6.4.2.3. Conclusions from Studies in Adults

Data obtained from the clinical study on the three individual notified probiotic strains showed that consumption of these 3 strains was well tolerated (Langkamp-Henken et al. 2015).

Regarding the other studies with *L. helveticus* Rosell-52, the reporting of adverse events (AEs) varied significantly between the studies. Either the authors generally stated that “no adverse events were observed” or “the preparation was well tolerated,” even when a criterion such as vomiting was part of the inclusion, or there are no safety data reported.

Only Vdovinchenko et al. (2008) and Song et al. (2010) discussed non-serious adverse events with Lacidofil®. Vdovinchenko et al. (2008) indicated that one patient in the probiotic group reported side effects (eructation and dry mouth), versus six in the control group, while Song (2010) noted mild abdominal pain in three patients and a skin eruption in one patient receiving Lacidofil®, but concluded that these adverse events could not be attributed to the use of Lacidofil®.

In conclusion, the absence of serious adverse events during clinical studies indicates that the strains *L. helveticus* Rosell®-52, *B. longum* ssp. *infantis* Rosell®-33 and *B. bifidum* Rosell®-71 are safe for use in adults.

6.4.3. Studies in Animals

6.4.3.1. Studies of *Probiokid*®

The research studies discussed below are summarized in Table 43 at the end of this section.

Cazzola et al. (2010a) investigated the impact of Probiokid® on immune system regulation in rats in T_h1 (cellular immune system) and T_h2 (humoral immune system) models. In the first experiment, 18 male Wistar rats weighing 200-225 g were randomly divided into 3 groups of 6 rats/group. The first group (vehicle group) was treated with physiological saline solution, the second was treated with vehicle and induced with *E. coli* on day 14 (control group), the third with Probiokid® and induced with *E. coli* on day 14 (T_h1 model).

After 17 days of treatment, the mean serum levels of IL-1α, IL-1β, IL-6, IFN-γ and TNF-α (pro-inflammatory modulators) was significantly lower in the Probiokid® group than in the control group ($p \leq 0.01$). Mean serum levels of the anti-inflammatory modulators IL-4 and IL-10 was significantly increased in the Probiokid® group compared with the control group ($p \leq 0.004$). Moreover the decrease in mass body weight of rats in the Probiokid® group was significantly smaller than in the control group ($p \leq 0.02$).

In a second experiment reported by Cazzola et al. (2010a), groups of 5 male Wistar rats weighing 200-250 g were orally administered saline solution (Groups 1 and 2) or Probiokid® (Group 3) daily for 10 days before and 4 days after injection of sterile saline solution (Group 1) or 3000 third-stage infective larvae of *N. brasiliensis* (Groups 2 and 3).

The level of pro-inflammatory modulators IL-1α, IL-1β, IL-6 and TNF-α was significantly decreased in the Probiokid® group compared with the control group ($p \leq 0.01$). There were no reports of adverse events during this study.

Huang et al. (2011) gavaged 10 healthy specific pathogen free BALB/c mice weighing 18-22 g with the combination of the three strains (1g/kg bw/day or about 3.3×10^9 cfu/kg bw/day) for 14 days. The microbiome of the synbiotic-fed mice and a control group of 10 mice that received only the saline carrier was compared before and after 14 days of gavage. There were no significant differences for *Enterobacteriaceae*, *Clostridium perfringens*, *Enterococci*, or *Lactobacilli* levels. However, there was a statistically significant increase in levels of *Bifidobacteria* after gavage with Probiokid®.

Table 43. Studies of *Probiokid*® in Animals.

References	Objectives	Study Design	Animal Model	Dose/Day	Duration	Safety-Related results
Cazzola et al. (2010a)	Investigate the impact of Probiokid® on the immune system regulation in rats in T_h1 (cellular immune system) and T_h2 (humoral immune system) models.	First experiment: 18 male Wistar rats divided in 3 groups (n=6/group): first with saline solution (vehicle), second with vehicle and induced with E. coli on day 14, and the third with Probiokid® and induced with E. coli on day 14. Second experiment: 6 groups of n=5 male Wistar rats were orally administered saline solution (group 1 and 2) or Probiokid® (group 3 to 6) for 10 days, then injected with either saline (group 1) or 3000 third-stage infective larvae of N. *brasiliensis* (groups 2 to 6)	Male Wistar Rats	Experiment 1 : 0.67 billion cfu/day Experiment 2 : 1 billion cfu/day	Experiment 1: 17 days Experiment 2: 14 days	In the two experiments the mean serum levels of pro-inflammatory modulators was significantly lower in the symbiotic group than in the control group ($p \leq 0.01$). In addition, in the first experiments, mean serum levels of the anti-inflammatory modulators were significantly increased in the Probiokid® group vs placebo group ($p \leq 0.004$). The decrease in mass body weight of rats in the Probiokid® group was significantly smaller than in the control group ($p \leq 0.02$). In the second experiment, Probiokid® reduced the level of circulating pro-inflammatory immune factors in T_h1 and T_h2 models of infection.
Huang et al. (2011)	Observe the effect of Probiokid® (Biostime [Probiokid®]) probiotics on mice intestinal microflora.	2 groups (n=10/group): negative control group (saline)/ probiotic group (1g/ kg Biostime [Probiokid®]) once/day	20 healthy specific pathogen free BALB/c mice	1g/kg/day or approx. $3.3x10^9$ cfu/kg/day	14 days	Significant increase in levels of *Bifidobacteria* after gavage with the symbiotic ($p \leq 0.05$). But no significant differences for *Enterobacteriaceae*, *Clostridium perfringens*, *Enterococci*, or *Lactobacilli* levels.

6.4.3.2. Studies of Individual Notified Strains

6.4.3.2.1. Studies of *Bifidobacterium bifidum* Rosell®-71 (R0071)

The research studies discussed below are summarized in Table 44 at the end of this section.

In broiler chickens, *B. bifidum* R0071 demonstrated the ability to reduce cellulitis, an infection of the connective tissue between the skin and the muscle generally due to *E. coli* infection of a skin wound, when compared to a control arm of broilers under the same conditions (Estrada et al. 2001). Examination of the cecal contents showed increased anaerobic microbes, specifically bifidobacteria, and a decrease in clostridia while aerobes and coliforms remained unchanged.

In a study in 45 male Sprague-Dawley rats weighing ~250 g, fed for 7 days, Dykstra et al. (2011) showed that orally ingested *B. bifidum* R0071 statistically significantly increased mucin-3 gene (MUC3) mRNA expression in both the jejunum and ileum. MUC3 expression did not change significantly in the proximal and distal colonic segments. This mucin is thought to be important for the adhesion of lactobacilli while at the same time it can reduce in viral attachment to epithelial cells in the small intestine and reduce viral particle replication.

Table 44. Studies of *B. bifidum* R0071 in Animals.

References	Objectives	Study Design	Animal Model	B. Bifidum dose	Duration	Safety-Related results
Estrada et al. (2001)	Evaluate the effect of *Bifidobacterium bifidum* on fecal bacterial populations, incidence of cellulitis, and growth performance in broiler chickens.	2 groups: Regular drinking water / Drinking water with B. *bifidum*	Broiler chickens	10 million *B. bifidum* cells/mL of drinking water	38 days	Chickens that received the treatment had statistically significantly increased numbers of fecal bifidobacteria on days 28 and 35 and there was a trend of reduction of total aerobic bacteria, coliforms, and clostridia. The number of condemnations for cellulitis and the total number of condemnations at slaughter were significantly reduced in the chickens with *B. bifidum.*
Dykstra et al. (2011)	Evaluation of probiotics administration to induce the repeated small intestinal Muc 3 expression in rats	*Lactobacillus plantarum* 299v (Lp299v), *Lactobacillus rhamnosus* R0011 (LrR0011), and *Bifidobacterium bifidum Rosell®-71 (R0071)* (BbR0071) were added repeatedly or intermittently to the drinking water of Sprague-Dawley rats	Male Sprague-Dawley rats	10 million or 1 billion *B. Bifidum* cells/ per day	7 days	Live Lp299v, BbR0071, and LrR0011 significantly increased Muc3 protein and mRNA expression in jejunum and ileum.

6.4.3.2.2. Studies of *Lactobacillus helveticus* Rosell®-52 (R0052)

The research studies discussed below are summarized in Table 45 at the end of this section.

Toxicity studies completed by Evic-Tox (Blanquefort, France) were performed *in vivo* with 20 6-7-week-old pathogen-free male and female Sprague-Dawley albino rats using *L. helveticus* Rosell®-52 (R0052). These studies involved 28-day gavage dosage of rats (high dose: 1-2x10^9 cfu/day). No toxicological symptoms or abnormalities were observed (EVIC 2005; unpublished).

The strain *L. helveticus* Rosell®-52 (R0052) has also been extensively marketed by Lallemand Health Solutions as a part of four following blends, described previously:

- Probio'Stick®, a combination of *L. helveticus* Rosell®-52 (R0052) (89.4%) and *B. longum* ssp. *longum* R0175 (10.6%).
- Lacidofil®, a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus* Rosell®-52 (R0052) (5%).
- Protecflor®, a combination of *B. longum* ssp. *longum* R0175 (33%), *L. helveticus* Rosell®-52 (R0052) (33%), *L. rhamnosus* R0011 (33%) and *S. boulardii* (125 mg).
- Oralis SB® powder, a combination of *L. helveticus* Rosell®-52 (R0052) (54 %), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%) and *S. cerevisiae* var *boulardii (7%).*

➢ **Animal Studies on Probio'Stick®**

To evaluate the role of Probio'Stick® on anxiety, Messaoudi et al. (2011) assessed its effects in the conditioned defensive burying test in the Wistar rat. In this test, rats exposed to a probe associated with a single foot shock show anxiety-related probe burying, head stretching, and approach/escape sequences towards the probe. Thirty-six male Wistar rats weighing ~200 g were randomly distributed into three groups of 12, which received Probio'Stick® (10^9 cfu/day), placebo, or diazepam. After an adaptation period, rats were placed inside a clear Plexiglas chamber equipped with a shock probe. When the animal touched the probe, a small electric shock was delivered and the rat's behavior was video-recorded for five minutes by experimenters unaware of the administered products. The percentage of approaches followed by escapes was then calculated (escapes/approaches x 100) in addition to a global stress/anxiety score determined by adding the ranks of duration of probe burying, head stretching, and percentage of approaches/escapes.

The stress/anxiety score was significantly lower in rats treated with Probio'Stick® or diazepam than with placebo ($p \leq 0.05$). Results were similar between Probio'Stick® and diazepam. No adverse events were reported during the 2-week study period.

Ait-Belgnaoui et al. (2014) investigated the effect of Probio'Stick® on anxiety-like markers induced by chronic psychological stress in mice. Male 6–8-week-old C57Bl6 mice weighing 21–23 g were used. Eight groups of 8–10 male mice received either saline or probiotic formulation (10^9 cfu/mouse/day) for 2 weeks. At the end of this

period, animals were submitted to water avoidance stress 1 hour/day for four consecutive days. The pretreatment with Probio'Stick® significantly attenuated hypothalamic-pituitary axis and autonomic nervous system activities in response to water avoidance stress, and reduced cFos expression in different brain areas. These central effects were associated with restoration of TJ barrier integrity in stressed mice (Ait-Belgnaoui et al. 2014). The authors did not report any adverse events.

Girard et al. (2009) assessed the effect of Probio'Stick® on apoptosis induced by the inflammatory condition observed after myocardial infarction (MI) in 10-week-old male Sprague-Dawley rats weighing 325-350 g. After an acclimatization period of 5 days, 35 rats were randomly distributed to one of two groups, Probio'Stick® (n=18) or placebo (n=17). A MI was then induced in both groups by occluding the left anterior descending coronary artery for 40 minutes. The animals were fed over a 4-week period and were killed following 3 days of reperfusion.

The levels of different proteins were measured: Bax/Bcl-2 (pro-apoptotic/anti-apoptotic) ratio and caspase-3 (pro-apoptotic) activity were reduced in the amygdala (lateral and medial), as well as in the dentate gyrus in the Probio'Stick® group when compared with the placebo ($p \leq 0.05$). Akt activity (anti-apoptotic) was statistically significantly increased in these same three regions. The authors did not report any adverse events.

Arseneault-Bréard et al. (2012) assessed the effect of Probio'Stick® on post-MI depression symptoms in Sprague-Dawley rats. Based on the hypothesis that probiotics achieve their effects through changes in intestinal permeability (which could have an effect on circulating pro-inflammatory cytokines), they also studied the effect of Probio'Stick® on intestinal barrier in the same rat model. Forty 12-week-old Sprague-Dawley rats weighing 325-350 g were randomly distributed in the following four groups: MI rats treated with Probio'Stick® (10^9 cfu/day) or vehicle and controls treated with Probio'Stick® (10^9 cfu/day) or vehicle. MI was induced in rats by occluding the left anterior descending coronary artery for 40 minutes. Rats in the control group underwent the same surgical procedure without actual coronary occlusion. Rats received either Probio'Stick® (n=20) or placebo (n=20) for 7 days before the surgical procedure and between the 7^{th} post-MI day and euthanasia.

Depressive behavior was assessed using the social interaction test, the forced swimming test, and the passive avoidance step-down test. Fluorescein isothiocyanate (FITC)-dextran was used to determine intestinal permeability. Plasma IL-β (pro-inflammatory modulator) levels were also quantified. In the 3 behavioral tests there was a significant interaction between MI and Probio'Stick®. Moreover, there was a statistically significant reduction of plasma IL-β concentrations in both Probio'Stick® groups compared with controls. No significant MI effect was observed in that case. A significant interaction between MI and Probio'Stick® was noted on FITC-dextran concentrations and concentrations were significantly increased in MI rats treated with vehicle compared to MI Probio'Stick® group. These results indicate a positive effect of Probio'Stick® on post-MI depression in rats.

Maintenance of the intestinal barrier integrity and a statistically significant decrease in plasma IL-β concentrations (compared with vehicle) was also observed with Probio'Stick® in rats after MI. This result reinforces the immune gut-brain axis

hypothesis to explain the efficacy of Probio'Stick® in depression symptoms. No adverse events were reported.

In 64 3-month-old male Sprague-Dawley rats weighing 325-350 g, Gilbert et al. (2012) conducted a study to determine if Probio'Stick® and n-3-rich diets, combined or alone, could be beneficial to attenuate post-MI depression-like behavior. Both a high PUFA n-3 diet and Probio'Stick® attenuated post-MI depression-like behavior ($p \leq 0.05$). Probio'Stick® significantly reduced caspase-3 activity in the dentate gyrus and medial amygdala ($p \leq 0.05$). The authors did not report any adverse events.

In a more recent study, Malick et al. (2015) ascertained vagus nerve involvement in the beneficial influence of probiotics on caspase activities in a post-MI animal model of depression. Forty-nine adult male Sprague-Dawley rats were randomly assigned to 8 groups, and Probio'Stick® and/or vehicle were administered daily for 14 days before myocardial infarction until euthanasia. Probio'Stick® significantly decreased caspase-8 activity with vagotomy. The authors concluded that the effect of probiotics on caspase activities in the amygdala after MI depends on an intact vagus nerve. No adverse events were reported associated with Probiostick®.

- **Animal Studies of Lacidofil®**

In a review of Lacidofil®, Foster et al. (2011) stated that, "Animal studies with Lacidofil® can be broadly classified into two main categories, those dealing with infection and those dealing with stress."

Studies in Rodent Models of Infection:

In the neonatal C57BL/6 mouse, *C. rodentium* infection causes severe diarrhea, weight loss, and eventually death. Daily pre-treatment with Lacidofil® for 7-22 days significantly reduced weight loss and death, epithelial cells hyperplasia, and *C. rodentium*-induced mucosal barrier dysfunction (Gareau et al. 2010). The effects of the probiotic were attributed to their ability to modulate the hypothalamus-pituitary-adrenal axis as serum corticosterone levels remained low in probiotic-treated animals. In addition, using either B-cell (JH -/-) or T-cell (rag1 -/-) deficient neonatal C57BL/6 mice, the authors showed that the protective properties of the probiotic was mediated via T-cells but not B-cells. There was no report of adverse events.

Johnson-Henry et al. (2004) demonstrated that pre-treatment with Lacidofil® before *H. pylori* challenge decreased the colonization of the gastric mucosa by the pathogen and reduced the gastric inflammation in 6-week-old female C57BL/6 mice. All of the mice treated with probiotics (n=20) remained healthy throughout the 9-week duration of the study. There were no signs of hair ruffling, weight loss, diarrhea, rectal prolapse, or loss of appetite.

In a study by Brzozowski et al. (2006), Lacidofil® was given to fifty 8-week-old male Mongolian gerbils weighing 30-50 g for 2 weeks after infection with *H. pylori*, and the outcomes were compared to gerbils given vehicle only or conventional triple eradication therapy. Both conventional therapy and Lacidofil® maintained gastric acid, plasma gastrin, and luminal somatostatin levels. Mucosal inflammation, gastric lesions, hyperplasia, and apoptotic body formation were completely eliminated by the conventional triple therapy and statistically significantly attenuated by Lacidofil®. The *H.*

pylori infected animals showed statistically significantly increased levels of COX-2 and BAX while Bcl-2 was significantly repressed; both conventional triple therapy and Lacidofil® given post-infection attenuated or eliminated these responses. No adverse events were reported.

Verdu et al. (2008) demonstrated that male BALB/c mice with chronic *H. pylori* infection had persistent behavioral and physiological changes even after the pathogen had been eradicated. These changes, such as delayed gastric emptying, increased intestinal permeability and increased gastric $CD3^+$ cell counts, leading to altered feeding behavior. The 6-8-week-old male mice in which the *H. pylori* had been eradicated were given either Lacidofil® or a placebo for 2 weeks. Those that received Lacidofil® had accelerated recovery of paracellular permeability compared with the placebo group, but the effect was modest. Probiotic treatment reduced inflammation compared to placebo ($p \leq 0.01$), and after two months resulted in faster gastric emptying ($p \leq 0.01$). The feeding patterns were also normalized in the Lacidofil® group, but not the placebo group. Thus, in this case, the changes in gastric emptying and feeding behavior did not appear to be mediated by an improvement in small intestine permeability. There was no report of adverse events.

Brzozowski et al. (2005) induced gastric ulceration in male Wistar rats by applying acetic acid directly to the anterior serosal surface of the stomach at the antro-oxyntic border. Eighty-four rats weighing 180-220 g were randomized and inoculated with *C. albicans* or saline. The rats were then treated with ranitidine, an anti-secretory agent, or acetylsalicylic acid (ASA) with or without Lacidofil® and recovery was monitored. In saline inoculated rats, the ulcers disappeared by day 25. Candida inoculation caused persistent ulceration, a fall in gastric blood flow and gastric acid output, and a rise in plasmic gastrin. Furthermore, inflammatory immune factors (IL-1β, TNF-α, EGF and TGF-α) were statistically significantly upregulated in the Candida-infected rats. The ranitidine and ASA treatments delayed the healing even further, but Lacidofil® reversed all the measured parameters to resemble uninfected, saline-inoculated rats. Lacidofil® significantly reduced the Candida colonization and suppressed the pro-inflammatory cytokine levels (IL-1β and TNF-α) thereby accelerating healing. The authors concluded that the probiotic was effective in the treatment of gastric ulceration and did not indicate any safety concerns.

A similar study was done in a rat model of colonic ulcerative colitis (Zwolinska-Wcisko et al. 2009). Trinitrobenzene sulfonic acid (TNBS) was rectally administered to 40 male Wistar rats weighing 180-220 g to induce colonic ulceration. The ulcerated rats were inoculated with *Candida* or with saline. These groups then received no treatment, Lacidofil®, or fluconazole for 9 days. The TNBS ulceration caused an increase in colonic weight due to inflammation, a decrease in colonic blood flow, and an increase in myeloperoxidase (MPO) levels (as a marker of colonic neutrophil infiltration). Groups that were inoculated with *Candida* showed delayed healing and elevated levels of plasma IL-1β and TNF-α. Administration of either fluconazole or Lacidofil® to the *Candida* infected rats significantly decreased the weight of the colon segments, the MPO activity and the IL-1β and TNF-α levels ($p \leq 0.05$). There was no significant difference between the fluconazole and Lacidofil® treatments; both were equally effective in minimizing the impact of *Candida*. There was no report of adverse events.

Studies in Rodent Models of Stress:

The impact of Lacidofil® on the brain-gut axis has been evaluated in four rodent models: two post-infection models and two psychological stress models. Gareau et al. (2011) demonstrated that 5-6-week-old female C57BL/6 and germ-free Swiss-Webster mice showed impaired memory when inoculated with a non-invasive pathogen, *C. rodentium*, and exposed to water avoidance stress. The germ-free mice showed memory loss upon infection even without applied stress. The behavior of these animals was evaluated using object recognition and T-maze testing. Anxiety was evaluated by light preference using a light/dark box, however, no change in anxiety was observed. The changes in memory persisted after the clearance of the *C. rodentium* and resolution of intestinal injury. In those mice treated with Lacidofil®, the colonic cell hyperplasia was restored and serum corticosterone and INF-γ levels were significantly ameliorated, but not the TNF-α level. In addition, exposure to the probiotic prevented a drop in expression of cFos and brain-derived neutrophilic factor in the CA1 hippocampus. Pre-treatment with Lacidofil® prevented the stress-induced memory deficits. No adverse effects were reported.

Using the psychological stress of maternal separation, Gareau et al. (2007) showed that neonatal (4-day-old) rat pups separated from the dam for 3 hours per day (n=6) had increased serum corticosterone and increased intestinal permeability when compared to sham controls (n=6). Administering Lacidofil® to the pups for 16 days normalized serum corticosterone level and gut permeability. There was no report of adverse events.

In another model of psychological stress, Zareie et al. (2006) applied water avoidance stress to 18 250-350-g adult male Brown Norway rats to determine if Lacidofil® could prevent stress-induced intestinal pathophysiology. The probiotic significantly reduced bacterial adherence to rat enterocytes in both the ileum and colon and eliminated bacterial translocation to the mesenteric lymph nodes, but did not prevent the increase in permeability. All rats receiving probiotics (n=8) remained healthy throughout the 17-day study. There were no signs of diarrhea, weight loss, or loss of appetite.

Smith et al. (2014) investigated in 4-week-old Rag1$^{-/-}$ and wild-type male and female C57BL/6 mice whether both behavior and intestinal function were modulated by the adaptive immune system and whether these changes were affected by psychological stress. Lacidofil® given for 4 weeks restored nonspatial memory, reduced anxiety-like behavior, and normalized colonic ion transport in Rag1$^{-/-}$ mice compared to placebo ($p \leq 0.05$). There was no report indicating the occurrence of adverse events.

Overall, these studies demonstrate that Lacidofil® reduced stress-induced responses, such as increased gut permeability, inflammation, and serum corticosterone levels. The impact of Lacidofil® seems to be mediated through the HPA-axis. No adverse events were reported in any of the animal studies performed with Lacidofil®.

- **Animal Studies of Protecflor®**

Protecflor® has been assessed in one *in vivo* study in a rat model of infection with *E. coli* (Bisson et al. 2009). Male Wistar rats weighing 200-225 g were divided into control (n=6) and Protecflor® (n=6) groups. Rats in the treatment group received $2x10^8$ cfu/day of Protecflor® while control rats were treated daily with a saline solution. Traveler's diarrhea was induced by oral administration of enterotoxigenic *E. coli* solution.

Treatments started 2 weeks before induction and continued for 3 days after, a total of 17 days. The induction of Levels of pro-inflammatory cytokines IL-1α, IL-6, IFN-γ and TNF-α were statistically significantly lower in the Protecflor® group compared with controls. Moreover, the secretion of anti-inflammatory cytokines IL-4 and IL-10 was significantly increased. Protecflor® also inhibited the production of diarrhea. Finally, Protecflor® also allowed animals to maintain their food and water consumption habits compared with controls. No adverse event was reported in this study.

- **Animal Studies of Oralis SB® Powder® (Commercialized Under the Brand Name Darolac® by Aristo Pharmaceutical)**

Mandal et al. (2015) reported on the evaluation of the effect of seven commercial symbiotics on kidney disease. Fifty-four male albino rats weighing 100±2.5 g were randomly divided into nine groups (n=6 rats/group). The control group received distilled water intraperitoneally for 7 days while the positive control group received 500 mg/kg bw/day acetaminophen intraperitoneally for 7 days. Seven commercially available symbiotic combinations were administered to the test groups at doses of 10^9 cfu/day for 3 weeks. Blood, kidney, liver, and stool samples were collected after scarification for biochemical tests, DNA fragmentation assays of kidney tissue, and kidney histological studies. Limited fecal analyses were conducted.

Blood urea nitrogen and toxicity indicators were significantly increased and antioxidant enzymes were significantly decreased by administration of acetaminophen. Blood urea nitrogen, toxicity indicators, glomerular necrosis, numbers of pathogenic bacteria, and DNA damage of kidney tissue were significantly reduced, and antioxidant enzymes were significantly increased, in the group receiving Oralis SB®. No adverse event was reported in this study.

Table 45. Studies of *L. helveticus* R0052 in animals.

Animal studies on *L. helveticus* R0052						
References	**Objectives**	**Study Design**	**Animal Model**	**L. helveticus dose**	**Duration**	**Safety-Related results**
EVIC (2005; unpublished)	Toxicity study	Subacute study of oral toxicity	Male SPF Sprague-Dawley albino rats	1 to 2 billion cfu/day	28 days	No toxicological symptoms or abnormalities were observed.
Animal studies on Probiostick: a combination of *L. helveticus Rosell®-52 (R0052)* (89.4%) and *B. longum ssp. Longum* R0175 (10.6%). Each sachet of ProbioStick® contains $3x10^9$ cfu, corresponding to $2.7x10^9$ cfu of *L. helveticus Rosell®-52 (R0052)*.						
References	**Objectives**	**Study Design**	**Animal Model**	**L. helveticus dose**	**Duration**	**Safety-Related results**
Girard et al. (2009)	Assess the effect of Probio'Stick® on the apoptosis induced by the inflammatory condition observed after myocardial infarction (MI) in rats.	Rats randomly distributed to two groups, probiotics (n=18) or placebo (n=17). A MI was induced in both groups by occluding the left anterior descending coronary artery for 40 minutes. The animals were fed over a 4-week period and were killed following 3 days of reperfusion.	10-week-old male Sprague-Dawley rats	0.894 billion cfu/ml	4 weeks	Proteins Bax/Bcl-2 (pro-apoptotic /anti-apoptotic) ratio and caspase-3 (pro-apoptotic) activity were reduced in the amygdala and dentate gyrus in the probiotics group ($p \leq 0.05$). Akt activity (anti-apoptotic) was significantly increased in these three regions. The authors did not report any adverse events.
Messaoudi et al. (2011)	Assess the effect of Probio'Stick® in the conditioned defensive burying test in the rats.	36 male Wistar rats were randomly distributed into three groups (n=12): probiotic preparation, placebo and diazepam as the reference substance	Male Wistar rats	0.894 billion cfu/day	2 weeks	Comparison of treated groups and controls showed a significant difference in the stress/anxiety score, which was lower in rats treated with Probio'Stick® and diazepam than with placebo ($p \leq 0.05$). Consumption of the PF containing *L. helveticus* R0052 and *B. longum* R0175 in combination mitigated psychological distress in three tests without displaying any adverse event.

Arsenault-Bréard et al. (2012)	Assess the effect of Probio'Stick® on post-MI depression symptoms in rats.	Forty Sprague-Dawley rats were randomly distributed in the following groups: MI rats treated with Probio'Stick® (10^9 cfu/day) or vehicle and controls treated with Probio'Stick® (10^9 cfu/day) or vehicle. MI was induced in rats by occluding the left anterior descending coronary artery for 40 minutes. Rats in the control group underwent the same surgical procedure without actual coronary occlusion.	Sprague-Dawley rats	0.894 billion cfu/day	7 days	In social interaction, forced swim, and passive avoidance step-down there was a significant interaction between MI and Probio'Stick® ($p \leq 0.05$). Significant reduction of plasma IL-β concentrations in both Probio'Stick® groups compared with controls ($p \leq 0.05$). No significant MI effect was observed. Significant interaction between MI and Probio'Stick® was noted on FITC-dextran concentrations and concentrations were significantly increased in MI rats treated with vehicle compared to MI Probio'Stick® group ($p \leq 0.05$). The authors did not report any adverse events.
Ait-Belgnaoui et al. (2014)	To investigate in mice the central effect of the probiotic combination with *B. longum* R0175 and *L. helveticus* R0052 on anxiety-like markers induced by chronic psychological stress	Eight groups of 8–10 male mice received orally during 2 weeks either saline or probiotic formulation (10^9 cfu/mouse/day). In parallel, the same experiments were conducted with another probiotic *L. salivarius* HA113 to verify the probiotic specificity on brain activity in response to chronic stress	Male 6–8 week old C57Bl6 mice	0.894 billion cfu/day	2 weeks	Pretreatment with the probiotic formulation attenuated hypothalamic-pituitary axis and autonomic nervous system activities in response to water avoidance stress, and reduced cFos expression in different brain area ($p \leq 0.05$). *L. salivarius* did not have these effects. R0175 and R0052 also prevented the water avoidance stress induced decrease hippocampal neurogenesis and expression changes in hypothalamic genes involved in synaptic plasticity. These central effects were associated with restoration of TJ barrier integrity in stressed mice ($p \leq 0.05$). No adverse events were reported.

Gilbert et al. (2012)	To determine if probiotics and n-3-rich diets, combined or alone, could be beneficial to attenuate post-MI depression-like behavior	64 rats divided in 4 groups: low or high n-3 PUFA diet, with or without Probio'Stick® (10^9 cfu/day)	64 male 3-month-old Sprague–Dawley rats	0.894 billion cfu/day	18 days	A high PUFA n-3 diet or probiotics attenuated post-MI depression-like behavior ($p \leq 0.05$). Probiotics significantly reduced caspase-3 activity in the dentate gyrus and medial amygdala ($p \leq 0.05$).
Malick et al. (2015)	to ascertain the vagus nerve involvement in the beneficial influence of probiotics on caspase activities in a post-MI animal model of depression	49 rats in one of 8 groups with myocardial infarction or sham, and with probiotic Probio'Stick® (10^9 cfu/day) or vehicle, and with or without vagotomy surgery	Adult male Sprague–Dawley rats	0.894 billion cfu/day	14 days before surgery and until euthanasia	Infarct size and caspase-3 were not affected by probiotics ($p>0.05$). Probiotics significantly decreases caspase-8 and caspase-3 activity only without vagotomy.
Animal studies on Lacidofil: a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus Rosell®-52 (R0052)* (5%). Each capsule of Lacidofil® contains $2x10^9$ cfu, corresponding to 10^8 cfu of *L. helveticus Rosell®-52 (R0052)*						
References	**Objectives**	**Study Design**	**Animal Model**	**L. helveticus dose**	**Duration**	**Safety-Related results**
Johnson-henry et al. (2004)	Determine if pretreatment of mice with probiotics suppresses colonization of *H. pylori*, strain SS1.	4 groups: Group A – sterile water Group B-probiotics Group C – challenged with *H. pylori* Group D – probiotics + challenged with *H. pylori*	30 6-week-old female C57BL/6 mice	50 million cfu/mL	9 weeks	All of the mice treated with probiotics (n=20) remained healthy throughout the duration of the study. There were no signs of hair ruffling, weight loss, diarrhea, rectal prolapse, or loss of appetite. Treatment significantly reduced incidence of *H. pylori* infection ($p \leq 0.2$) and reduced incidence of moderate-severe inflammation ($p \leq 0.14$)

Brzozowski et al. (2005)	Evaluate the effects of ranitidine, aspirin with or without probiotic on gastric secretions and gastric ulcers after a *Candida* infection	4 groups: Group A – inoculated with *C. albicans* or saline Group B – *C. albicans* + aspirin Group C – *C. albicans* + ranitidine Group D – *L. acidophilus* or Flumycon, with or without live *C. albicans* + aspirin or ranitidine	84 male Wistar rats	1 million cfu/mL	25 days	Significant decrease of percent of mice infected with C. albicans when treated with probiotics ($p \leq 0.05$).
Brzozowski et al. (2006)	Effect of H. pylori treatment with triple therapy or probiotics on gastric acid secretion	Group A: triple therapy with omeprazole, amoxicillin, imidazole Group B: Lacidophilin ($2x10^7$ cfu; (0.4ml/animal i.g.)	50 male Mongolian gerbils	-	2 weeks	There was no report of adverse events. Both treatment prevent reductions in basal gastric acid and mucosal changes.
Zareie et al. (2006)	Effect of probiotics on preventing stress-induced intestinal pathophysiology	4 groups: 10 days of water avoidance stress (WAS) or sham stress, with or without probiotic pre-treatment for 7 days before and during tests	18 male Brown Norway Rats	50 million cfu/mL	17 days	All rats receiving probiotics remained healthy throughout the study. There were no signs of diarrhea, weight loss, or loss of appetite. Animal weight increased continuously in the rats subjected to sham stress, or remained relatively constant in rats exposed to WAS. Absence of normal weight gain in animals exposed to WAS was not affected by probiotic treatment. Probiotic treatment prevents pathophysiology of stress, including bacterial adherence to colonic and ileal tissue ($p \leq 0.05$)
Gareau et al. (2007)	Effect of probiotics on maternal separation (MS) – induced gut dysfunction	4 groups: maternally-separated (MS) or not-separated (NS) mice, given vehicle or probiotic (10^8 cfu, BID)	12 neo-natal (7-day-old) C57BL/6 rat pups	10 million cfu/day	16 days	Administering Lacidofil® to the pups normalized all the serum corticosterone and the gut permeability ($p \leq 0.05$).

Verdu et al. (2008)	Investigate if the antigenic or bacterial content of the gut influences the rate of recovery of host physiology induced by chronic *H. pylori* infection after bacterial eradication.	3 groups: Probiotic for 2 weeks (100 µL of 10^{10} cfu Lacidofil), placebo for 2 weeks, or crude *Hp* antigen weekly for 2 months	91 male BALB/c mice	-	2 weeks	There was no report of adverse events. Probiotic treatment reduced inflammation compared to placebo ($p \leq 0.01$), and after two months resulted in faster gastric emptying ($p \leq 0.01$)
Zwolinska-Wcisko et al. (2009)	Effect of probiotics or antifungal (flucanozole) on healing of ulcerative colitis, along with trinitrobenzene sulfonic acid	4 groups: group A; vehicle (saline) group B: C. albicans group C: C. albicans + 10^8 cfu/ml Lacidofil group d: C. albicans + fluconazole	40 male Wistar rats	5 million cfu/ml	9 days	No adverse events were reported. Both treatments significantly reduced plasma IL-1B and TNF-a ($p \leq 0.05$)
Gareau et al. (2010)	Characterize Citrobacter rodentium infection in neonatal mice and effect of probiotics in improving disease severity	4 groups: probiotic Lacodifil (10 µL; 10^8 cfus) or maltodextrin, and challenged with *C. rodentium* or vehicle	Neonatal mice from timed-pregnant C57BL/6 mice	5 million cfu/day OR 50 million cfu/ml	7 days	C. rodentium infection in newborn mice causes death and probiotics promote survival ($p \leq 0.05$), but only in the presence of T cells. There was no report of adverse events.
Gareau et al. (2011)	Effect of *C. rodentium* infection on anxiety-like behavior and memory, and whether probiotics provide protective benefits	Infected with *C. rodentium* or not, treatment with probiotics (Lacodofil 10^9 cfu/mL) or placebo maltodextrin, and subjected to Water avoidance stress (WAS) or not	C57BL/6 mice and germ-free Swiss-Webster mice	0.3 billion cfu/day	17 days OR 37 days	No adverse events were reported. Probiotic treatment prevented stress-induced memory deficits, increases to serum corticosterone, and other signs of infections, compared to placebo ($p \leq 0.05$)

Smith et al. (2014)	to determine whether both behavior and intestinal function were modulated by the adaptive immune system and whether these changes were affected by psychological stress	Treated with Lacidophil (10^9 cfu/ml) or placebo maltodextrin, subject to Water avoidance stress (WAS) and behavioral tests	Male and female 6-8 wk old $Rag1^{-/-}$ and wild-type C57BL/6 mice	0.3 billion cfu/day	4 weeks	No adverse events were reported. Probiotics restores nonspatial memory, reduces anxiety-like behavior, and normalizes colonic ion transport in $Rag1^{-/-\ mice}$, compared to placebo ($p \leq 0.05$).

Animal studies on Protecflor®: a combination of *B. longum* ssp. *longum* R0175 (33%), *L. helveticus Rosell®-52 (R0052)* (33%), *L. rhamnosus* R0011 (33%) and *S. boulardii* (125 mg). Each capsule of Protecflor® contains $5x10^9$ cfu of bacteria, corresponding to $1.67x10^9$ cfu of *L. helveticus Rosell®-52 (R0052)*.

References	Objectives	Study Design	Animal Model	L. helveticus dose	Duration	Safety-Related results
Bisson et al. (2009)	Assess Protecflor® in a rat model for travelers' diarrhea with *E. Coli*	5 groups: group A – positive placebo (vehicle + infection) group B – negative placebo (vehicle + no infection) group C – FFI (Protectflor strains without the yeast - $8x10^8$ cfu/day) + infection group D – *S. boulardii* ($2x10^8$ cfu/day) + infection group E – Protectflor ($8x10^8$ cfu/day bacteria and $2x10^8$ cfu/day of yeast) + infection Treatment for 2 weeks before and infection, and 3 days after.	30 Male Wistar rats weigh-ing 200-225 g	0.264 billion cfu/day	17 days	Level of pro-inflammatory cytokines were significantly lower in the Protecflor® group and the secretion of anti-inflammatory cytokines was significantly increased ($p \leq .05$) than infected, vehicle treated rats. No adverse event was reported. No significant differences in weight between treatment groups before infection induced.

Animal studies on Oralis SB® powder: a combination of *L. helveticus Rosell®-52 (R0052)* (54 %), *L. rhamnosus* R0011 (29%), *B. longum ssp. longum* R0175 (10%) and *S. cerevisiae var boulardii (7%).* Each sachet of Oralis SB® powder contains $1.25x10^9$ cfu of bacteria, corresponding to $6.7x10^8$ cfu of *L. helveticus Rosell®-52 (R0052)*						
References	**Objectives**	**Study Design**	**Animal Model**	**L. helveticus dose**	**Duration**	**Safety-Related results**
Mandal et al. (2015)	Evaluation the effect of Oralis on kidney disease (induced by acetaminophen)_	9 groups: Group 1: control group (water) Group 2: Positive control (water and acetaminophen) Groups 3 to 9: 7 different commercial synbiotics (10^9 cfus/day), including group 4: Darolac	54 Albino male rats	0.54 billion cfus/day	3 weeks	No adverse event was reported in this study. Treatment by Darolac reduced renal damage.

6.4.3.3. Conclusions from Studies in Animals

The three notified probiotic strains *L. helveticus* Rosell®-52, *B. longum* ssp. *infantis* Rosell®-33, and *B. bifidum* Rosell®-71 have been widely studied, both alone and in combination, in a variety of animal species including chickens and rodents (mice, rats, and gerbils). In none of these studies has administration of probiotics evidenced indications of toxicity or pathogenicity.

6.5. Safety Evaluations by Authoritative Bodies

6.5.1. Authoritative Evaluations of *L. helveticus* Rosell®-52 (R0052)

As previously noted, the strain *Lactobacillus helveticus* was first described by Orla-Jensen in 1919 (Naser et al. 2006). It can be isolated from sour milk and cheese, particularly Emmental and Gruyère cheese (Bergey's Manual of Systematic bacteriology 1986). It is a lactic acid bacterium (LAB) used for centuries in fermented food and has been well studied.

The *Lactobacillus* genus appears in the partial list of microorganism compiled by the Food and Drug Administration in 2001. It is provided as an example of harmless lactic acid bacteria that have been prior sanctioned, which indicates that FDA views the genus as safe (FDA 2001).

The International Dairy Federation (IDF), in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Salminen et al. 2002). The species *Lactobacillus helveticus* is listed in this inventory. Since 2007, *Lactobacillus helveticus* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authorities (EFSA Journal 2015), a status that has been maintained through each annual update.

In Canada, the Natural Health Products Regulations of 2004 classified probiotics under the definition of Natural Health Products. In its probiotics monograph, the Natural and Non-Prescription Health Products directorate of Health Canada listed *Lactobacillus helveticus* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible for generic health claims as well in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009). This list included the *acidophilus* species but did not include *L. helveticus*. However, after assessment of the characterization profile of the specific strain R0052, *L. helveticus* R0052 received a letter of non-objection to be used in foods (usually issued for a non-novel food status) and to benefit from the applicable regulatory provisions (letter in Appendix II).

Similarly, the specific strain *Lactobacillus helveticus* R0052 has been approved for use in functional foods in Brazil (RESOLUÇÃO - RE N° 2.123, DE 30 DE MAIO DE 2014), as well as in Australia where the TGA (Therapeutic Goods Administration) listed it on its website as an equivalent to *L. acidophilus* R0052 (Appendix III).

L. helveticus is also included in the list of "Substances that may typically be considered to be a health supplement" in South Africa (Medicines Control Council 2014). Food Safety and Standards Authority of India has recognized *Lactobacillus helveticus* and added it to the List of Strains as Probiotics (Schedule –X of the Food Safety and

Standards Regulation - No. 1-4/Nutraceutical/FSSAI-2013). In Korea, this strain has been referenced in the Health Functional Food Code (2010).

In China, *Lactobacillus helveticus*, is included in the positive list of strains (Appendix VI).

As aforementioned, the *Lactobacillus helveticus* R0052 strain was obtained by Lallemand Health Solutions (formerly known as Institut Rosell) in 1990. It is a proprietary culture provided to Institut Rosell by Weinstein Nutritional Products of California, USA. *Lactobacillus helveticus* R0052 was isolated in 1990 from a dairy culture. The strain is deposited in the "Collection Nationale de Cultures de Microorganismes" (CNCM) at Pasteur Institute in France, which guarantees having an isolate of the strain in a safe and secure place at all times.

Lactobacillus helveticus Rosell®-52 (R0052) has been sold worldwide for many years, as a powder, or as a part of the following blends:

- Probio'Stick®, a combination of *L. helveticus* Rosell®-52 (R0052) (89.4%) and *B. longum* ssp. *longum* R0175 (10.6%).
- Probiokid®, a combination of *L. helveticus* Rosell®-52 (R0052) (80%), *B. longum* ssp. *infantis* Rosell®-33 (R0033) (10%) and *B. bifidum* Rosell®-71 (R0071) (10%).
- Lacidofil®, a combination of *L. rhamnosus* R0011 (95%) and *L. helveticus* Rosell®-52 (R0052) (5%).
- Protecflor®, a combination of *B longum* ssp. *longum* R0175 (33%), *L. helveticus* Rosell®-52 (R0052) (33%), *L. rhamnosus* R0011 (33%) and *S. boulardii* (125 mg).
- Oralis SB® powder® a combination of *L. helveticus* Rosell®-52 (R0052) (54 %), *L. rhamnosus* R0011 (29%), *B. longum* ssp. *longum* R0175 (10%) and *S. cerevisiae var boulardii (7%).*

Probio'Stick® was first marketed in 2006 in Canada and Poland and has since been sold in other countries in the worldwide. In Canada, Probio'Stick® ® is a natural product approved by the Natural and Non-Prescription Health Product Directorate of Health Canada with the non-traditional health claims, which means after in-depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 80021343).

Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime. Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, United Kingdom, etc... In Canada, Probiokid® is a natural product approved by the Natural and Non-Prescription Health Product Directorate of Health Canada with approved non-traditional health claims, which means after conducting an in depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 80019993).

The first marketing authorization for Lacidofil® was obtained in Ukraine in 1995, and the product has since been sold in other countries in the worldwide. In Canada, Lacidofil® is a natural product registered by the Natural and Non-Prescription Health Products Directorate of Health Canada with approved non-traditional health claims, which means after conduction an in depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 02246224).

The first launch of Protecflor® was in 2007 in Australia, France, India, Serbia and Montenegro,UK, and the product has since been sold in other countries in the worldwide... In Canada, Protecflor® is a natural product approved by the Natural Health Products Directorate of Health Canada with approved non-traditional health claims, which means after conducting an in depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 80021342).

The first launch of Oralis SB® powder® was in India under the Brand name Darolac® by Aristo Pharmaceutical. In Canada, Oralis SB® powder® is a natural health product approved by the Natural and Non-Prescription Health Products Directorate of Health Canada with non-traditional health claims, which means after in depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 80064421).

Additionally, *L. helveticus* Rosell®-52 (R0052) has also been extensively and widely marketed by Lallemand Health Solutions as a combination (with other strains) in more than 170 other formulas in more than 20 different countries (USA, Canada, UK, France, Italy, Spain, Sweden, Poland, India, China, Australia, South Africa, Mexico, Malaysia, Indonesia, etc.). Moreover, this strain has also been purchased in more than 10 countries by subcontractors who used them in various probiotic formulas.

L. helveticus Rosell®-52 (R0052) appears on a peer-reviewed list of strains that has been recognized for their probiotic properties in different scientific reviews (Mercenier et al. 2002, Johnson-Henry et al. 2004).

*All Natural Product Number (NPN) can be found with relevant details on the publicly accessible database of licensed finished products on Health Canada's website: https://health-products.canada.ca/lnhpd-bdpsnh/index-eng.jsp

6.5.2. Authoritative Evaluations of *B. longum* ssp. *infantis* Rosell®-33 (R0033)

Bifidobacteria predominate in the intestinal tract shortly after birth. They are important normal constituents of the human gastrointestinal microflora and occur at concentrations of 10^9 to 10^{10} cells/g of feces (Tanaka et al. 2000). *B. bifidum* is a natural inhabitant of the intestinal tract microbiota and is a lactic acid bacterium (LAB) which has been used for many years in fermented food. Tanaka et al. (2000) noted that many communities consume fermented milk products that contain high numbers of lactic acid producing bacteria. Bifidobacteria have been part of human nutrition for centuries and are now more and more being introduced into many fermented dairy food products and supplements.

The International Dairy Federation (IDF), in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Salminen et al. 2002). The species *B. infantis* is listed on this inventory. *B. infantis* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authorities (EFSA Journal 2015) during each year of the QPS listing.

In Canada, the Natural Health Products Regulations of 2004 classified probiotics under the definition of Natural Health Products. On its probiotics monograph, the Natural and Non-Prescription Health Products Directorate (NNHPD) of Health Canada listed *B. longum* ssp. *infantis* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible to generic health claims as well in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009) allowing freely its use without the need to pre-marketing oversight, which in other terms reflects an established safe history of use. The particular strain *B. longum* ssp. *infantis* R0033 as a single strain finished product is approved by the NNHPD under NPN* 80040500.

The Australian Therapeutic Goods Administration (TGA) includes *B. infantis* on the “List of approved substances that can be used as Active ingredients in “Listed” Medicines.” *B. longum* ssp *infantis* is also included in the list of “Substances that may typically considered to be a health supplement” in South Africa (Medicines Control Council. 2014).

Food Safety and Standards Authority of India has also recognized the strain *B. infantis* and added it in the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). Additionally, in China, this strain is included in the positive list of strain.

B. longum ssp. *infantis* Rosell®-33 (R0033) (*B. infantis* ATCC 17930) was isolated by G. Reuter from infant intestine and received by Lallemand Health Solutions (formerly known as Institut Rosell) in March 1988. The strain is deposited in the “*Collection Nationale de Cultures de Microorganismes*” (CNCM) at Pasteur Institute, France, which guarantees to have an isolate of the strain in a safe and secure place at all times.

B. longum ssp. *infantis* Rosell®-33 (R0033) has been sold worldwide for many years, as a powder, or as a part of Probiokid®, a combination of *L. helveticus* Rosell®-52 (R0052) (80%), *B. longum* ssp. *infantis* Rosell®-33 (R0033)(10%) and *B. bifidum* Rosell®-71 (R0071) (10%). Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime. Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, United Kingdom, etc... In Canada, Probiokid® is a natural product approved by the Natural and Non-Prescription Health Products Directorate of Health Canada with non-

traditional health claims, which means after conducting an in depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 80019993).

Additionally, *B. longum* ssp. *infantis* Rosell®-33 (R0033) has also been extensively marketed by Lallemand Health Solutions as a combination (with other strains) in more than 40 other formulas.

*All Natural Product Number (NPN) can be found with relevant details on the publicly accessible database of licensed finished products on Health Canada's website: https://health-products.canada.ca/lnhpd-bdpsnh/index-eng.jsp

6.5.3. Authoritative Evaluations of *B. bifidum* Rosell®-71 (R0071)

Bifidobacteria predominate in the intestinal tract shortly after birth. They are important normal constituents of the human gastrointestinal microflora and occur at concentrations of 10^9 to 10^{10} cells/g of feces (Tanaka et al. 2000). *Bifidobacterium bifidum* is a natural inhabitant of the intestinal tract microbiota and is a lactic acid bacterium (LAB) which has been used for many years in fermented food. Tanaka et al. (2000) noted that many communities consume fermented milk products that contain high numbers of lactic acid producing bacteria. Bifidobacteria have been part of human nutrition for centuries and are now more and more being introduced into many fermented dairy food products and supplements.

The International Dairy Federation (IDF), in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Salminen et al. 2002). The species *B. bifidum* is listed on this inventory. Since 2007, *B. bifidum* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authorities (EFSA Journal 2015).

In Canada, the Natural Health Products Regulations of 2004 classified probiotics under the definition of Natural Health Products. On its probiotics monograph, the Natural and Non-Prescription Health Products directorate of Health Canada listed *B. bifidum* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible to generic health claims as well in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009). *B. bifidum* has been included which allows its use without pre-marketing oversight and means that it has an established safe history of use. Particularly, *B. bifidum* Rosell®-71 (R0071) as a single strain finished product has been evaluated in its country of origin (Canada) by the Natural and Non-Prescription Health Products directorate of Health Canada for its safety, quality, and efficacy and is consequently approved with Non-traditional health claim (NPN* 80063177).

The Australian Therapeutic Goods Administration (TGA) includes *B. bifidum* on the "List of approved substances that can be used as Active ingredients in "Listed" Medicines." *B. bifidum* is also included in the list of "Substances that may typically be considered to

be a health supplement" in South Africa (Medicines Control Council 2014). Food Safety and Standards Authority of India, has also recognized the strain *B. bifidum* and added it in the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). In Korea, *B. bifidum* has been referenced in the Health Functional Food Code (2010).

The *Bifidobacterium bifidum* Rosell®-71 (R0071) strain was obtained in 1988 from the Dutch company DSM. It was isolated from an adult intestine by G. Reuter. The strain is deposited in the "*Collection Nationale de Cultures de Microorganismes*" (CNCM) at Pasteur Institute, which guarantees to have an isolate of the strain in a safe and secure place at all times.

B. bifidum Rosell®-71 (R0071) has been sold worldwide for many years, as a powder, or as a part of Probiokid®, a combination of *L. helveticus* Rosell®-52 (R0052) (80%), *B. longum* ssp. *infantis* Rosell®-33 (R0033) (10%) and *B. bifidum* Rosell®-71 (R0071) (10%). Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime.Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, United Kingdom, etc... In Canada, Probiokid® is a natural product approved by the Natural Health Product Directory of Health Canada with the non-traditional health claims, which means after an in depth assessment of safety, quality, and efficacy of the strains and the finished product (NPN* 80019993).

Bifidobacterium bifidum Rosell®-71 (R0071) has also been extensively marketed by Lallemand Health Solutions as a combination (with other strains) in more than 80 other formulas.

*All Natural Product Number (NPN) can be found with relevant details on the publicly accessible database of licensed finished products on Health Canada's website: https://health-products.canada.ca/lnhpd-bdpsnh/index-eng.jsp

6.5.4. Authoritative Evaluations of Probiokid®

As noted previously, the three notified probiotic strains *L. helveticus* Rosell®-52 (R0052), *B. longum* ssp. *infantis* Rosell®-33 (R0033), and *B. bifidum* Rosell®-71 (R0071) have long been consumed at the same ratios, 80:10:10, in the product Probiokid®. Probiokid® was first launched as a natural health product in China and its marketing started in January 2002, under the trade name Biostime.

Since 2002, more than 1.2 billion Probiokid® sachets have been sold in several countries.

In Canada, Probiokid® is a natural product approved by the Natural and Non-Prescription Health Product Directorate of Health Canada (NNHPD) with the following approved non-traditional health claims (NPN* 80019993).

- *"Helps to reinforce the body's natural defenses in children"*
- *"Participates in healthy microflora balance"*

- *"Probiotic to benefit health and/or confer a health benefit"*
- *Source of probiotics in infants*

This means that the NNHPD conducted an in depth assessment of safety, quality, and efficacy of the strains and the blend, in children and infants, as well as a review of Lallemand Health Solutions research and methodology before approving the product. This approval reflects Health Canada's recognition of these enumerated points.

Moreover, Probiokid® is included in an ongoing program of pharmacovigilance for monitoring adverse events. Probiokid® has also been the subject of a Periodic Safety Update Report (PSUR) which outlines the safety profile of this product. The first PSUR covers the period from 01 January 2006 through 31 December 2012.During that time, a total of 574,205,358 sachets of Probiokid® were sold globally and 4 non-serious adverse events were reported. Actually, the PSUR covering the period from 01 January 2013 through 31 December 2016, a total of 564 810 364 sachets of Probiokid® were sold. No non-serious or serious adverse events have been reported.

Additionally, there were no actions for safety reasons initiated by any Health Authority concerning Probiokid® sachets. A regular review of the published scientific literature detected no reports of adverse events related to the intake of Probiokid® or one of the following strains *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) and *Bifidobacterium bifidum* Rosell®-71 (R0071).

The global evaluation of the safety of Probiokid® sachets during the reporting period did not point out significant information to be notified on the safety profile of the product.

*All Natural Product Number (NPN) can be found with relevant details on the publicly accessible database of licensed finished products on Health Canada's website: https://health-products.canada.ca/lnhpd-bdpsnh/index-eng.jsp

6.6. Decision-Tree Analysis of the Safety of the Three Notified Strains

The decision tree published by Pariza et al. (2015) indicates that all three notified strains, *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum ssp. infantis* Rosell®-33 (R0033) *Bifidobacterium bifidum* Rosell®-71 (R0071), "are deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption" (Pariza et al. 2015).

The responses to each of the questions asked in the decision tree are as follows for all three strains:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? YES

2. Has the strain genome been sequenced? YES

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? YES

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? YES

5. Does the strain produce antimicrobial substances? NO

6. Has the strain been genetically modified using rDNA techniques? NO

7. Was the strain isolated from a food that has a history of safe consumption for which the species to which the strain belongs is a substantial and characterizing component (not simply an 'incidental isolate')? YES for *L. helveticus* Rosell®-52; NO for the other two strains, which were isolated from healthy human intestinal mucosa

8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? NO

6.7. Safety Assessment and GRAS determination

6.7.1. Introduction

This section presents an assessment that demonstrates that the intended use of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) *Bifidobacterium bifidum* Rosell®-71 (R0071), and their combination at a respective ratio of 80:10:10, in term infant formula is safe and is GRAS.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of each strain individually and as a blend at a respective ratio of 80:10:10 is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of term infants to these strains as a blend and individually through their intended use in milk-based infant formula is not harmful. In the second step, the intended use of the three strains as a blend and individually and is determined to be GRAS by demonstrating that the safety of these probiotics under their intended conditions of use is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether the intended use of a substance (or organism) is GRAS, in accordance with Section 281(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified

scientific experts. This “common knowledge” element of a GRAS determination consists of two components:

1. data and information relied upon to establish the scientific element of safety must be generally available; and
2. there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the addition of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), *Bifidobacterium bifidum* Rosell®-71 (R0071) and their combination at a respective ratio of 80:10:10 to infant formula is safe and is GRAS.

6.7.2. Safety Evaluation

Several convergent lines of evidence support the conclusion that the intended use of the three strains, *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), *Bifidobacterium bifidum* Rosell®-71 (R0071), individually and in an 80:10:10 blend is safe. The strains are members of genera, *Lactobacillus* and *Bifidobacterium*, that have long been consumed by humans both a food contaminants and as microorganisms used in food processing. The specific species, *L. helveticus, B. longum* (ssp. *infantis*), and *B. bifidum*, also have long histories of safe consumption and all have Qualified Presumption of Safety (QPS) status in the European Union as well as equivalent safety recognition in numerous other countries.

The bacterial strains, Rosell®-52, Rosell®-33, and Rosell®-71, have been widely consumed as probiotics worldwide for many years, both individually and combined in the product Probiokid® and other combination products.

The three strains have been subjected to tests of minimum inhibitory concentration (MIC) to establishing phenotypic resistance to clinically significant antibiotics and have been found not to exhibit resistance above established microbiological breakpoints. They have moderate levels of binding capacity, do not produce biogenic amines, and do not produce antibiotics. Neither of the bifidobacteria produces D-lactate; *L. helveticus* forms 41% L- and 59% D-lactate.

The genomes of all three strains have been sequenced and fully annotated; the annotations indicate that they do not harbor virulence genes, potentially transferable antimicrobial resistance, decarboxylase capable of forming biogenic amines, or genes encoding production of antibiotics.

The three bacterial strains have been studied in infants and children, both healthy and compromised with conditions such as diarrhea, thrush, and rotavirus infection. This research includes one large-scale study of the three individual strains given to about 50 infants each at doses of $3x10^9$ cfu/day for 8 weeks, in which the primary endpoint was

growth (unaffected) and secondary endpoints were tolerance and adverse events (not significantly different from controls). The research also includes twelve studies in which Probiokid® (80% *L. helveticus*, 10% *B. longum* ssp. *infantis*, 10% *B. bifidum*) was given to 656 infants and children at doses as high as $2x10^{10}$ cfu/day and for durations as long as 9 months. Additionally, in 15 studies, 522 infants and children suffering from diarrhea, dysbiosis, *H. pylori* infection, atopic dermatitis, or *C. difficile* infection, were treated with Lacidofil® (a preparation that includes *L. helveticus*) at doses up to $6x10^{8}$ cfu/day for up to 3 months. Lastly, another preparation containing L. helveticus, Oralis SB®, was given for 14 days to 110 children with dental issues in 3 studies, with doses up to $1.34x10^{9}$ cfu/day. In none of these studies were issues of intolerance or adverse reactions reported differing in nature, frequency, or severity from controls.

In addition to the studies in infants and children which provide the primary clinical evidence for the safety of the intended use of *L. helveticus, B. longum* ssp. *infantis*, and *B. bifidum* and Probiokid® as probiotics to be added to infant formula, there is an extensive body of research in healthy and compromised adults and in animals, all of which confirms the safety of the strains.

Finally, a decision-tree analysis based on Pariza et al. (2015) indicated that the three strains “are deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.”

6.7.3. General Recognition of Safety

The intended use of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), *Bifidobacterium bifidum* Rosell®-71 (R0071) and their 80:10:10 blend, to be added to milk-based infant formula intended for consumption by term infants, has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by establishing the identity and probiotic characteristics of the strains, demonstrating their freedom from pathogenic or other risk factors, and concluding that the expected exposure to *L. helveticus* Rosell®-52 (R0052), *B. longum* ssp. *infantis* Rosell®-33 (R0033), *B. bifidum* Rosell®-71 (R0071) and their 80:10:10 blend by infants is without significant risk of harm. Finally, because this safety assessment is based on generally available (published) data, and so satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the addition of *Lactobacillus helveticus* Rosell®-52 (R0052), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033), *Bifidobacterium bifidum* Rosell®-71 (R0071) individually or in an 80:10:10 blend to milk-based term-infant formula has been made through the deliberations of an Expert Panel consisting of Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., who reviewed this monograph, prepared by Lallemand Health Solutions and edited by JHeimbach LLC, as well as other information available to them. Dr. James T.

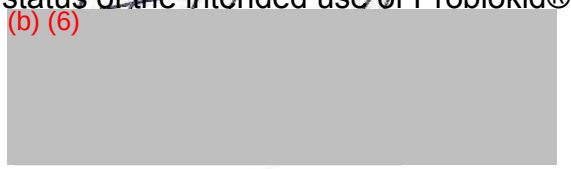
Heimbach served as Scientific Advisor to the Panel. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients, including probiotic bacteria, intended for addition to infant formula. They critically reviewed and evaluated the publicly available information and the potential exposure to *Lactobacillus helveticus* Rosell®-52 (R0052) strain, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) strain, *Bifidobacterium bifidum* Rosell®-71 (R0071) strain, and their blend at a respective ratio of 80:10:10, anticipated to result from its intended use, and individually and collectively concluded that no evidence exists in the available information on *Lactobacillus helveticus* Rosell®-52 (R0052) strain, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) strain, *Bifidobacterium bifidum* Rosell®-71 *(R0071)* strain, and their combination at a respective ratio of 80:10:10, that demonstrates, or suggests reasonable grounds to suspect, a hazard to term infants under the intended conditions of use *Lactobacillus helveticus Rosell®-52 (R0052)* strain, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) strain, *Bifidobacterium bifidum* Rosell®-71 (R0071) strain, and their 80:10:10 blend.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, the intended use of *Lactobacillus helveticus* Rosell®-52 (R0052) strain, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) strain, *Bifidobacterium bifidum* Rosell®-71 (R0071) strain, and their 80:10:10 blend, is GRAS by scientific procedures.

6.8. Statement Regarding Information Inconsistent with GRAS

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with our conclusion of GRAS status of the intended use of Probiokid® or the three individual bacterial strains.

(b) (6)



PART 7. LIST OF SUPPORTING DATA AND INFORMATION

Sections:
7.1. Generally Available (Published) Documents
7.2. Generally Available but Unpublished Government Documents
7.3. Unpublished Documents

7.1. Generally Available (Published):

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APPENDIX I - HEALTH CANADA

Use(s) or Purpose(s)
Statement to the effect of

Medicinal ingredients from Appendix I, Table 1, 2, and 3
Source of Probiotics.

Medicinal ingredients from Appendix I, Table 1, 2, and 3 except *Lactobacillus crispatus* and *Lactobacillus gallinarum*

Helps support intestinal/gastrointestinal health (Alonso and Guarner 2013; DuPont and DuPont 2011; WGOGG 2011; Rolfe 2000).
Could promote a favorable gut flora (Bezkorovainy 2001; Morelli 2000; Collins et al. 1998).

Table 1: Medicinal Ingredients - BACTERIA

Proper and Common Names	References
For "source of probiotics" claim only Return to Table 3 footnote 1 referrer	
Bifidobacterium adolescentis	Masco et al. 2004; Skerman et al. 1980
Bifidobacterium animalis (including *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*)	Masco et al. 2004; Skerman et al. 1980
Bifidobacterium bifidum	Skerman et al. 1980
Bifidobacterium breve	Skerman et al. 1980
Bifidobacterium longum (including *Bifidobacterium longum* ssp. *infantis, Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *suis*)	Mattarelli et al. 2008
Lactobacillus acidophilus	Johnson et al. 1980; Skerman et al. 1980
Lactobacillus amylolyticus	Validation List No. 68 1998
Lactobacillus amylovorus	Nakamura 1981
Lactobacillus brevis	Skerman et al. 1980
Lactobacillus buchneri	Skerman et al. 1980
Lactobacillus casei	JCICSB 2008; Skerman et al. 1980
Lactobacillus coryniformis	Skerman et al. 1980
Lactobacillus crispatus Table 3 Footnote1	Skerman et al. 1980
Lactobacillus curvatus	Skerman et al. 1980
Lactobacillus delbrueckii (including *Lactobacillus delbrueckii* ssp. *bulgaricus* & *Lactobacillus delbrueckii* ssp. *delbrueckii*)	Beijerinck 1901; Howey et al. 1990
Lactobacillus farciminis	Validation List no. 11, 1983
Lactobacillus fermentum	Skerman et al. 1980
Lactobacillus gallinarum Table 3 Footnote1	Fujisawa et al. 1992
Lactobacillus gasseri	Validation List No. 4 1980

Table 1: Medicinal Ingredients - BACTERIA	
Lactobacillus helveticus	Skerman et al. 1980
Lactobacillus hilgardii	Skerman et al. 1980
Lactobacillus johnsonii	Fujisawa et al. 1992
Lactobacillus kefiranofaciens	Fujisawa et al. 1988
Lactobacillus kefiri	Validation List no. 11, 1983
Lactobacillus mucosae	Roos et al. 2000
Lactobacillus panis	Wiese et al. 1996
Lactobacillus paracasei	JCICSB 2008; Collins et al. 1989
Lactobacillus paraplantarum	Curk et al. 1996
Lactobacillus plantarum	Skerman et al. 1980
Lactobacillus pontis	Vogel et al. 1994
Lactobacillus reuteri	Validation List No. 8, 1982
Lactobacillus rhamnosus	Collins et al. 1989
Lactobacillus salivarius	Skerman et al. 1980
Lactobacillus sanfranciscensis	Validation List no. 16, 1984b
Lactococcus lactis	Validation List no. 20, 1985
Leuconostoc citreum	Farrow et al. 1989
Leuconostoc pseudomesenteroides	Farrow et al. 1989
Leuconostoc lactis	Skerman et al. 1980
Leuconostoc mesenteroides	Skerman et al. 1980
Oenococcus oeni	Dicks et al. 1995
Pediococcus acidilactici	Skerman et al. 1980
Pediococcus pentosaceus	Skerman et al. 1980
Propionibacterium freudenreichii (including *Propionibacterium freudenreichii* ssp. *shermanii*)	Skerman et al. 1980
Propionibacterium acidipropionici	Skerman et al. 1980

APPENDIX II - HEALTH CANADA - RECLASSIFICATION OF STRAIN R0052

Health Canada / Santé Canada
Health Products and Food Branch / Direction générale des produits de santé et des aliments

Bureau of Nutritional Sciences
Nutrition Evaluation Division
Food Directorate
Sir Frederick G Banting Research Centre
Tunney's Pasture,
Ottawa, Ontario

August 11, 2010

Mrs Solange Henoud
Regulatory Affairs
Institut Rosell-Lallemand
8480 Boul. Saint-Laurent
Montreal, Qc, H2P 2M6
Tel: (514) 858-4617
Fax: (514) 383-4493

Subject: Taxonomical reclassification for *Lactobacillus helveticus* strain R0052 and its marketing as probiotic for use in food

Dear Mrs. Henoud,

This is in response to the questions raised at the meeting that was held with Lallemand, Inc. on January 15th, prepared with input from the Novel Food Section. The meeting was attended by both Novel Food Section and Nutrition Labelling and Claims Section of the Food Directorate. The company representative has requested an opinion on:

1. Acceptance of the taxonomical reclassification of a bacterial strain previously known as *Lactobacillus acidophilus* R0052 to *Lactobacillus helveticus* R0052.
2. Acceptance for the marketing of *Lactobacillus helveticus* R0052 *strain* as a probiotic in foods

1. Acceptance of the taxonomical reclassification of a bacterial strain previously known as *Lactobacillus acidophilus R0052* to *Lactobacillus helveticus* R0052.

The Bureau of Microbial Hazards (BMH) has received a submission from Rosell-Lallemand presenting the evidence from both traditional typing methods, such as biochemical, genetic, and growth characterization, and contemporary advanced molecular and genomic methods supporting the taxonomical reclassification of a bacterial strain previously known as *Lactobacillus acidophilus* R0052 to *Lactobacillus helveticus* R0052. BMH has conducted an evaluation of the data submitted and concluded that the data reviewed in this submission support

.../2

Canada

the taxonomical reclassification of the bacterial strain *Lactobacillus acidophilus* R0052 to *Lactobacillus helveticus* R0052. BMH offers no objection to the change of name for this strain and noted no special safety concerns with respect to antimicrobial resistance of *L. helveticus* R0052.

2. Rosell-Lallemand wishes to continue marketing the *Lactobacillus helveticus* R0052 strain as a probiotic for use in food

We have no objection to the use of *Lactobacillus helveticus* R0052 as a probiotic in food, provided that the manufacturer follows the conditions for the use of probiotic claims in foods as outlined in the *Guidance Document-The use of Probiotic Microorganisms in Food* (**http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/probiotics_guidance-orientation_probiotiques-eng.php**). Consistent with the guidance provided in the above mentioned document we would like to remind you that the Nutrition Labelling and Claims section (NLC) does not review data supporting the claim "probiotic" unless it is a disease risk reduction or therapeutic type claim and as such requires a premarket submission, or upon a voluntary request from a manufacturer to review a specific "probiotic" claim. Please note that for submitting a request for the evaluation of a specific claim the manufacturer is required to follow the guidance provided in the *Guidance Document for Preparing a Submission for Food Health Claims* (http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/health-claims_guidance-orientation_allegations-sante-eng.php). In addition, when a "probiotic" claim is made, labelling guidance in section 8.7.1 of the CFIA *Guide to Food Labelling and Advertising* (http://www.inspection.gc.ca/english/fssa/labeti/guide/tab8e.shtml) should be followed.

Thank you again for your inquiry and we hope you find this information helpful.

(b) (6)

Lydia Dumais
Section Head
Nutrition Labelling and Claims
Nutrition Evaluation Division
613-954-0632

cc. Hélène Couture, Bureau of Microbial Hazards

INSTITUT ROSELL
LALLEMAND

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Taxonomic identification of Lactobacillus Rosell 52
By Thomas A. Tompkins, Ph.D, Biological and Microbiology Research Director – October 2010

The Lactobacillus strain Rosell 52 (R0052) was isolated from naturally fermented sweet acidophilus milk in 1990 by Dr Brochu at Institut Rosell. At that time, the tools available for taxonomic identification were based on morphology (microscopical observation) and phenotypic characterization. By using these tools, R0052 strain was classified as L. acidophilus.

Since then, molecular methods based on the sequencing of variable regions of the genome have been gradually developed. This led to the subdivision of the previous L. acidophilus species into a number of new and novelspecies : *L. acidophilus, L. amylovorus, L. crispatus, L. gallinarum, L. gasseri and L. johnsonii.* These species
could be differentiated by a yery slight variation (1- 5 bp) in the sequence of the VI hypervariable region in the16s rDNA gene. The corresponding sequence of R0052 was determined and compared to type strains of each of the species of the *L. acidophilus* complex. Based on these data. it was confirmed that R0052 is closely related to
the *L. acidophilus* group A complex and also with *L. helveticus* and *L. amylovorus*. Il was found eventually that VI sequencing did not alloy sufficient discrimination among strains belonging to this group.
By comparison to other sequenced genes, such as ITS, a close homology was always found between R0052 and *L. acidophilus* group A complex strains, as well as with *L. helveticus* and *L. suntoryeus* strains.

Recently, a DNA-DNA hybridation study displayed a stronger homology (ca 80 %) between R0052 and the type strains of *L. helveticus* and *L. suntoryeus* than between R0052 and members of the *L. acidophilus* group A complex (ca. 50-60%).
Moreover, the sequencing of the Slp (S-layer protein) gene and other more conservated
genes confirmed the strong homology between R0052 and both *L. helveficus* and *L. suntoryeus*. The latter species has also been ultimately re-classified as: *L. helveticus* (Naser et al 2006).

Thus, R0052 was re-designated as *L. helveticus* and a number of subsequent investigations using a variety of techniques, including whole genome sequencing, have confirmed the identification. The draft genome of R0052 has been submitted to GenBank (accession number: GDSub12155 WGS genome submission for Project # 51767) and the manuscript describing the genome of R0052 is in progress and we hope to publish this manuscript in 2011. Furthermore, the genome sequence analysis of the current working strain of R0052 was compared to the original version of the strain deposited in the culture collection of the Institut Pasteur in 199. This work was performed independently by Genome Quebec and they did not observe any significant variation

indicating that this strain has retained all of its original properties (internal report from Genome Quebec). So despite the name change, the microbe remains identical.

In conclusion, the strain R0052. initially identified as a member of the *L. acidophilus* group A complex, and still very closely related to it both genotypically and phenotypically, should be considered as a *L. helveticus* strain after close evaluation by using the current molecular taxonomy techniques.

This reclassification does not imply any difference in the intrinsic properties of the strain. and does not contradict any of the data obtained with R0052, especially with respect to the safety and efficacy. It is just for the sake of scientific rigor and consistency that future publications on this strain will mention *L. helveticus* as the taxonomic identity of R0052.

Today, the evidence supporting the role of *L. helveticus* as a probiotic (see attached Table) is surpassing the evidence of *L. acidophilus* (Sanders, 2007). Furthermore, a new investigation of bacteria associated with the human gut microbiome in healthy populations has shown that *L. helveticus* is the most commonly occurring member of the *lactobacilli* which reside in the intestinal tract (Qin et al 2010). Thus, this new information supports the concept that *L. helveticus* should be used as a probiotic to promote a healthy and balanced microbiome.

References:

Cachat, E. & Priest, F. G. (2005). Lactobacillus suntoryeus sp. nov.,isolated from malt whisky distilleries. Int J Syst Evol Microbiol 55, 31–34.

Food and Agriculture Organization/World Health Organization. (2001). Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Available from: http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm.

Naser S.M., Hagen K.E., Vancanneyt M., Cleenwerck I., Swings J. and Tompkins T.A. (2006) Reclassification of Lactobacillus suntoryeus Cachat and Priest 2005 as a later synonym of Lactobacillus helveticus (Orla-Jensen 1919) Bergey et al. 1925 (Approved Lists 1980). Int. J. Syst. Evol. Microbiol. 56(2):355-360.

Qin J., Li R., Raes J. et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464.

Sanders M.E, Hamilton J., Reid G. and Gibson G (2007) A nonviable preparation of Lactobacillus acidophilus is not a probiotic. Clin. Infect. Dis. 44(6): 886.

Table of Articles Showing Probiotic Potential of L. helveticus

Bacteriocins: R0052 has 3 genes encoding bacteriocins (helveticin J + 2 novel bacteriocins). Helveticin J is also found in L. acidophilus NCFM.

Atassi F, Brassart D, Grob P, Graf F, Servin AL. (2006) *In vitro* antibacterial activity of *Lactobacillus helveticus* strain KS300 [R0052] against diarrhoeagenic, uropathogenic and vaginosis-associated bacteria. J Appl Microbiol. 101(3):647-54.

Bonadè A, Murelli F, Vescovo M, Scolari G. (2001) Partial characterization of a bacteriocin produced by *Lactobacillus helveticus*. Lett Appl Microbiol. 33(2):153-8.

Thompson JK, Collins MA, Mercer WD. (1996) Characterization of a proteinaceous antimicrobial produced by *Lactobacillus helveticus* CNRZ450. J Appl Bacteriol. 80(3):338-48.
Vaughan EE, Daly C, Fitzgerald GF. (1992) Identification and characterization of helveticin V-1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. J Appl Bacteriol. 73(4):299-308.
Joerger MC, Klaenhammer TR. (1990) Cloning, expression, and nucleotide sequence of the Lactobacillus helveticus 481 gene encoding the bacteriocin helveticin J. J Bacteriol. 1990 Nov;172(11):6339-47.
Upreti GC, Hinsdill RD. (1973) Isolation and characterization of a bacteriocin from a homofermentative Lactobacillus. Antimicrob Agents Chemother. 4(4):487-94.

Intestinal monitoring showing *L. helveticus* survive gastrointestinal transit.

Saito Y, Sakamoto M, Takizawa S, Benno Y. (2004) Monitoring the cell number and viability of *Lactobacillus helveticus* GCL1001 in human feces by PCR methods. FEMS Microbiol Lett. 231(1):125-30.
Shinoda T, Kusuda D, Ishida Y, Ikeda N, Kaneko K, Masuda O, Yamamoto N. (2001) Survival of *Lactobacillus helveticus* strain CP53 in the human gastrointestinal tract. Lett Appl Microbiol. 32(2):108-13.
Firmesse O, Mogenet A, Bresson JL, Corthier G, Furet JP. (2008) *Lactobacillus rhamnosus* R11 consumed in a food supplement survived human digestive transit without modifying microbiota equilibrium as assessed by real-time polymerase chain reaction. J Mol Microbiol Biotechnol. 14(1-3):90-9.

***In vitro* demonstration of the direct impact of *L. helveticus* R0052 on human cell lines.**

Jandu N, Zeng ZJ, Johnson-Henry KC, Sherman PM. (2009) Probiotics prevent enterohaemorrhagic Escherichia coli O157:H7-mediated inhibition of interferon-gamma-induced tyrosine phosphorylation of STAT-1. Microbiol. 155(Pt 2):531-540.
Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM. (2007) Surface-layer protein extracts from Lactobacillus helveticus inhibit enterohaemorrhagic Escherichia coli O157:H7 adhesion to epithelial cells. Cell Microbiol. 9(2):356-367.
Atassi F, Brassart D, Grob P, Graf F, Servin AL. (2006) In vitro antibacterial activity of *Lactobacillus helveticus* strain KS300 [R0052] against diarrhoeagenic, uropathogenic and vaginosis-associated bacteria. J Appl Microbiol. 101(3):647-54.
Sherman PM, Johnson-Henry KC, Yeung HP, Ngo PS, Goulet J, Tompkins TA. (2005) Probiotics reduce enterohemorrhagic Escherichia coli O157:H7- and enteropathogenic E. coli O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. Infect Immun. 73(8):5183-5188.
Wallace TD, Bradley S, Buckley ND, Green-Johnson JM. (2003) Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. J Food Prot. 66(3):466-72.
Easo JG, Measham JD, Munroe J and Green-Johnson JM (2002) Immunostimulatory actions of Lactobacilli: Mitogenic induction of antibody production and spleen cell proliferation by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*. Food & Agricultural Immunology 14: 73-83.

APPENDIX III – TGA AUSTRALIA

Substances that may be used in listed medicines in Australia – Therapeutic Goods Administration
Ingredient summary

TGA eBS

Ingredient Summary

Ingredient Name	Lactobacillus helveticus
Category	Approved Biological Name
Synonyms	Lactobacillus acidophilus strain R52 Lactobacillus acidophilus R52
Purpose	Ingredient purpose not held on file
CAS Number	
Reference	International Journal of Food Microbiology
Restrictions	Ingredient restrictions not held on file
Use	Can be used as an Active ingredient for Export Only; Listed Medicines; Over The Counter; Solely for Export; Ingredient sub type: Can be used as an Excipient ingredient for Prescription Medicines;

END OF SUMMARY

Ingredient Summary

TGA-Complementary Medecine Evaluation Committee-

Extracted Ratified Minutes Seventy Third Meeting (pages 8 and 9)

Australian Government
Department of Health and Ageing
Therapeutic Goods Administration

CMEC 73
COMPLEMENTARY MEDICINES
EVALUATION COMMITTEE

EXTRACTED RATIFIED MINUTES
SEVENTY THIRD MEETING
4 SEPTEMBER 2009

Abbreviations

ADRAC Adverse Drug Reactions Advisory Committee
ADRs Adverse Drug Reactions
ARTG Australian Register of Therapeutic Goods
BMI Body Mass Index
CMEC Complementary Medicines Evaluation Committee
IBS Irritable bowel syndrome
IRCH International Regulatory Cooperation for Herbal medicines
NH&MRC National Health and Medical Research Council
NICM National Institute of Complementary Medicine
OCM Office of Complementary Medicines
OICG Office of Complementary Medicines Industry Consultation Group
OLS Office of Legal Services
OMSM Office of Medicines Safety Monitoring
TCM Traditional Chinese Medicine
TGA Therapeutic Goods Administration
UC Ulcerative colitis

The Complementary Medicines Evaluation Committee (CMEC) held its seventy third meeting at the Melbourne Hilton Airport Hotel from 9.30 a.m. to 4.40 p.m. on Friday 4th September 2009.

8.1 10.2 *L. helveticus* as a new substance

Background

A TGA Officer introduced this item by advising Members that a sponsor had notified the TGA of the taxonomic reclassification of *Lactobacilus acidophilus R52.* This substance, which has been supplied in Australia for ten years, has been re-named '*L. helveticus'* based on the results of recently completed genetic studies

The sponsor has indicated that it intended to seek approval of *L. helveticus* as a new substance for use in Listed probiotic medicines. Although comprehensive scientific evidence was provided for evaluation to substantiate the reclassification, the sponsor sought a decision regarding the necessity of re-evaluating the safety of the substance.

The OCM had obtained advice from the TGA's Office of Laboratories and Scientific Services (OLSS) in relation to:

the validity of taxonomic reclassification of *L. acidophilus* strain Rosell 52 (R52) as a strain of *L. helveticus*;

the introduction of *L. helveticus* strain as an approved new substance for use in Listed probiotic medicines; and

on whether a microbiological safety evaluation would be necessary for this strain of *L. helveticus* in order to allow probiotic medicines currently Listed in the ARTG to contain the new strain.

The Acting Chief Microbiologist recommended to the OCM that where the reason for an application for use of a new '*Lactobacillus*' substance is purely the result of a taxonomic change to the name of a bacterium, and there has been no change to the actual strain of a bacterium used in production of presently Listed probiotic medicines, then it would be reasonable for the TGA to adopt a flexible approach to its consideration of the application for a new ingredient, waiving the requirement for the safety review.

The OCM has acted on this recommendation and are in the process of updating the code tables in then Electronic Listing Facility.

Discussion

Members noted the re-listing of *L. acidophilus R52* as the substance *L. helveticus*. Members also noted that a full safety review was not considered necessary.

OUTCOME

CMEC noted the TGA decision, that due to the taxonomic reclassification of *Lactobacillus acidophilus* strain Rosell 52 (R52) as a strain of *Lactobacillus helveticus*; and will be permitted for use in Listed probiotic medicines.

APPENDIX IV – MOH CHINA

Notice Regarding "the List of Bacterial Species Allowed for Food Application" Issued by the General Administrative Office of
Minister of Health, People's Republic of China

MOH office Notice (2010) No. 65

To Department of Health of all the provinces, autonomous regions, Direct-controlled municipalities, Xinjiang Production and Construction Corps, Chinese Disease Control Center, National Center for Health Inspection and Supervision:
In accordance with "Food Safety Law" and the relevant regulations on its implementation, we organized and established "the list of bacterial species allowed for food application" and issued it herewith. Please Comply with it.

Enclosure: The list of bacterial species allowed for food application

April 22nd, 2011

Bacterial Species Allowed for Food Application

	Name	**Latin Name**
(1)	*Bifidobacterium*	*Bifidobacterium*
1	*Bifidobacterium adolescentis*	*Bifidobacterium adolescentis*
2	*Bifidobacterium animalis (Bifidobacterium lactis)*	*Bifidobacterium animalis (Bifidobacterium lactis)*
3	*Bifidobacterium bifidum*	*Bifidobacterium bifidum*
4	*Bifidobacterium breve*	*Bifidobacterium breve*
5	*Bifidobacterium infantis*	*Bifidobacterium infantis*
6	*Bifidobacterium longum*	*Bifidobacterium longum*
(2)	*Lactobacillus*	*Lactobacillus*
1	*Lactobacillus acidophilus*	*Lactobacillus acidophilus*
2	*Lactobacillus casei*	*Lactobacillus casei*
3	*Lactobacillus crispatus*	*Lactobacillus crispatus*
4	*Lactobacillus delbrueckii* ssp. *Bulgaricus (Lactobacillus bulgaricus)*	*Lactobacillus delbrueckii* ssp. *Bulgaricus (Lactobacillus bulgaricus)*
5	*Lactobacillus delbrueckii* ssp. *lactis*	*Lactobacillus delbrueckii* ssp. *lactis*
6	*Lactobacillus fermentium*	*Lactobacillus fermentium*
7	*Lactobacillus gasseri*	*Lactobacillus gasseri*
8	*Lactobacillus helveticus*	*Lactobacillus helveticus*
9	*Lactobacillus johnsonii*	*Lactobacillus johnsonii*
10	*Lactobacillus paracasei*	*Lactobacillus paracasei*
11	*Lactobacillus plantarum*	*Lactobacillus plantarum*
12	*Lactobacillus reuteri*	*Lactobacillus reuteri*
13	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*

14	*Lactobacillus salivarius*	*Lactobacillus salivarius*
(3)	*Streptococcus*	*Streptococcus*
1	*Streptococcus thermophilus*	*Streptococcus thermophilus*

Note: 1. The bacterial species that have been used in food manufacturing and processing can be used continuously. The new species which are not listed here should comply the “New Resource Food Regulation”;

2. The list of bacterial species allowed for baby food application will be issued separately.

Institute of Microbiology, Chinese Academy of Sciences

Identification Test Report

Report Number: (2009) Microbiological Test Number: 066
Name of Sample: Freeze-Dried Bacterial Powder
Test Item: Microorganism Specie Identification
Trustor: Beijing Representative Office of Canada Lallemand Inc.

April 15th, 2009

Institute of Microbiology, Chinese Academy of Sciences
Identification Test Report

(2009) Microbial test No. 066

Trustor: Beijing Representative Office of Canada Lallemand Inc.
Sample Name: Freeze Dried Bacterial Powder (Strain: L. acidophilus-R0052)
Quantity of sample: 1 strain Sample submission date: March 2009
Person in charge of identification test (signature): Zhou Yuguang
Person conducting the identification test (signature): Liu Yingfu
Identification test contents and results: (The present identification test results is only valid for the submitted sample, without permission, the name of the test institution cannot be used for commercial promotion)

Identification Test Conclusion:

In our laboratory settings, based on the 16S rDNA sequence data, morphology, physiological and biochemical data, the bacteria sample submitted by Beijing Representative Office of Canada Lallemand Inc was identified as:

Lactobacillus acidophilus

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Institute of Microbiology, Chinese Academy of Sciences
Identification Test Report
(2009) Microbiological test No. 066
Trustor: Beijing Representative Office of Canada Lallemand Inc.

1. Biochemical Test Results:

Test Items	Results	Test Items	Results
Gram Stain	Positive	Carbohydrate producing acid (continued)	
Cell Shape	Rod	Ribose	-
Spore formation	-	Trehalose	+
Catalase	-	Xylose	-
Hydrogen peroxide	-	Rhamnose	-
Growth in the air	+	Maltose	+
Growth at 45°C	-	Lactose	+
Growth at 15°C	-	Melitose	-
Growth with 6.5NaCl %	-	Sorbitol	-
Growth at pH9.6	-	Melibiose	-
Growth at pH4.5	+	Galactose	+
Carbohydrate producing acid		Mannitol	-
Glucose	+	Arabinose	-
Mannose	+	Sodium gluconate	-
Melizitose	-	Sucrose	+
Fructose	+	Cellobiose	+
Salicin	+	Esculin	+
Amygdalin	+		

2. 16S rDNA Sequencing Results:

TACCGGCGTGCCTTATACATGCAGTCGAGCGAGCAGAACCAGCAGATTTACTTCGGTAATGACGCTGGGGACGCGAGCGGCGG
ATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTAGGATACCACTTGGAAACAGGTGCTAATACCGGATAATAAAGCAG
ATCGCATGATCAGCTTATAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAAC
GGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAA
GTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAATTGCATCGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTC
CATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGC
TCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCC
GCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTAGTGCCATCCT
AAGAGATTAGGAGTTCCCTTCGGGGACGCTAAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATTATTAGTTGCCAGCATTAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGTACAACGAGAAGCG
AGCCTGCGAAGGCAAGCGAATCTCTGAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCG
CTAGTAATCGCGGATCAGAACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTCTGCAA
TGCCCAAAGCCGGTGGCCTAACCTTCGGGAAGGAGCCGTCTAAGACAGT

Institute of Microbiology, Chinese Academy of Sciences

Identification Test Report

Report Number: (2009) Microbiological Test Number: 067
Name of Sample: Freeze-Dried Bacterial Powder
Test Item: Microorganism Specie Identification
Trustor: Beijing Representative Office of Canada Lallemand Inc.

April 15th, 2009

Institute of Microbiology, Chinese Academy of Sciences
Identification Test Report

(2009) Microbial test No. 067

Trustor: Beijing Representative Office of Canada Lallemand Inc.
Sample Name: Freeze Dried Bacterial Powder (Strain: L. acidophilus-R0052)
Quantity of sample: 1 strain Sample submission date: March 2009
Person in charge of identification test (signature): Zhou Yuguang
Person conducting the identification test (signature): Liu Yingfu
Identification test contents and results (This identification test results is only valid for the submitted sample, without permission, the name of the test institution cannot be used for commercial promotion)

Identification Test Conclusion:

In our laboratory settings, based on the multilocus sequence typing analysis results, the bacteria sample (strain: L.acidophilus-R0052) submitted by Beijing Representative Office of Canada Lallemand Inc was identified as:

Lactobacillus helveticus

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Institute of Microbiology, Chinese Academy of Sciences
Stamped of Identification test center, Institute of Microbiology, Chinese Academy of Sciences
April 15th, 2009

Institute of Microbiology Chinese Academy of Sciences
Identification Test Report

(2009) Microbial test No. 067

Trustor: Beijing Representative Office of Canada Lallemand Inc.

1. Biochemical Test Results:

Test Items	Results	Test Items	Results
Gram Stain	Positive	Carbohydrate producing acid (continued)	
Cell Shape	Rod	Ribose	-
Spore formation	-	Trehalose	+
Catalase	-	Xylose	-
Hydrogen peroxide	-	Rhamnose	-
Growth in the air	+	Maltose	+
Growth at 45°C	-	Lactose	+
Growth at 15°C	-	Melitose	-
Growth with 6.5NaCl %	-	Sorbitol	-
Growth at pH9.6	-	Melibiose	-
Growth at pH4.5	+	Galactose	+
Carbohydrate producing acid		Mannitol	-
Glucose	+	Arabinose	-
Mannose	+	Sodium gluconate	-
Melizitose	-	Sucrose	+
Fructose	+	Cellobiose	+
Salicin	+	Esculin	+
Amygdalin	+		

2. 16S rDNA sequencing results:

3. atpA Gene Sequencing Results

TTACGTTCGGGAGAGGGATCGCCCGGGCTCACGGTTCTTAATAATGCATTGGCCGGTGAGTTATTAAAATTTGATAACGGTTC
ATACGGTATCGCTCAAAACTTGGACTCTAGTGATGTAGGTATCATCATTTTGGGTCAATTTGACCATATCCGTGAAGGTGACC
GTGTTCAAAGAACTGGTCGAATTATGTCAGTTCCTGTTGGTGACGCCTTAATCGGTAGAGTAGTTAACCCATTGGGTCAACCA
GTTGACGGTTTAGGTGAAATTAAATCTGATAAGACACGTCCTATTGAAGAAAAAGCTCCAGGGGTTATGGACCGTCAATCAGT
TAACCAACCACTTCAAACTGGTATTAAAGCTATTGATGCCTTAGTTCCAATTGGTCGTGGACAGCGTGAATTGATTATTGGTG
ACCGTAAAACTGGTAAGACTAGTTTAGCCATCGATACAATTCTTAACCAAAAGAACCAAGACGTAATTTGTATTTATGTTGCT
ATTGGTCAAAAGGAATCAACCGTTAGAACTCAAGTAGAAACTTTAAAGCGTTTCGGAGCAATGGATTACACCATTGTTGTTGA
AGCGGGCCCAAGTGAACCAGCCCCAATGTTATACATTGCACCATATGCTGGTACTGCTATGGGTGAGGAATTTATGTACAATG
GCAAGGATGTATTAATCGTATTTGACGACCTATCTAAGCAAGCCGTCGCTTACCGTGAACTTTCTTTGCTTCTCCGTCGTCCG
CCTGGTCGTGAAGCTTACCCAGGTGATGTCTTCTACTTACACTCACGTTTACTTGAACGTAGTGCTAAGTTGAGTGACAAACT
TGGTGGTGGATCATTGACTGCATTGCCAATTATCCAAACTGAAGCGGGAGATATTTCTGCATATATTCCAACCAACGTAATTT
CTATTACTGATGGTCAGATTTTCTTACAAAGTGATTTGTTCTTTGCTGGTACTCGTCCAGCCATTGATGCTGGTAACTCAGTT
TCTCGTGTTGGTGGTAACGCCCAGATAAAGGCTATGAAGAAAGTTGCTGGTACTTTACGTTACTGACCTTGCTGCTAATCGTG
AACTTGAAAGTTTGCTCAATTGGGAGTGGATCTTGAATCAGCCCCTTCAAGCCAAACTT

4. GroEL Gene Sequencing Results:

TTTGAAGGGATGAAGAACGTCACTGCTGGTGCTAACCCAGTTGGTATTCGTCGCGGTATTGAAAAGGCAACTAAGGCTGCTGT
TGATGAACTTCACAAGATCAGCCACAAGGTTGAATCAAAGGATCAAATTGCTAACGTTGCTGCTGTTTCATCAGCTTCTAAAG
AAGTTGGTGCTTTGATCGCTGATGCTATGGAAAAGGTTGGTCACGATGGTGTTATCACTATTGAAGACTCACGTGGTATCAAC
ACTGAACTTTCAGTAGTTGAAGGTATGCAATTTGACCGTGGTTACTTATCACAATACATGGTAACTGACAACGACAAGATGGA
AGCAGACCTTGATAATCCATACATCTTGATCACTGACAAGAAGATTTCAAACATTCAAGACATCTTGCCATTACTTCAAGAAA
TTGTTCAACAAGGCAAGTCATTGTTGATCATTGCTGACGATGTAACTGGTGAAGCTTTTCCAACTCTTGTTTTGAACAAGATC
CGTGGGGTACAA

5. Tuf Gene Sequencing Results:

GCCCACGTAGAATACGAAACTGAAAATCGTCACTACGCTCACATGGACGCTCCAGGCCACGCCGACTACATCAAGAACATGAT
TACCGGTGCTGCACAAATGGATGGGGCCATCTTAGTTGTTGCTGCAACTGATGGTCCTATGCCACAAACTCGTGAACACATTT
TGCTTGCTCGTCAAGTTGGTGTTAACTACATCGTAGTATTCTTGAACAAGTGCGATTTAGTTGACGACCCAGAGTTGATCGAC
TTGGTTGAAATGGAAGTTCGTGACTTGTTAACTGAATACGATTACCCTGGTGACGATATTCCAGTTGTTCGTGGTTCAGCTTT
GAAGGCTTTACAAGGCGACAAGGAAGCTCAAGAACAAATTCTTAAGTTAATGGACATCGTTGATGAATACATCCCAACTCCAG
AACGTCAAACTGACAAGCCATTCTTAATGCCAGTTGAAGACGTATTCACTATCACTGGTCGTGGTACTGTTGCTTCAGGTCGT
ATCGACCGTGGTACTGTTAAGGTCGGCGATGAAGTTGAAATCGTTGGTTTGGTAGACAAGGTTCTTAAGTCAGTTGTTACTGG
TTTGGAAATGTTCCACAAGACTTTGGACTTAGGTGAAGCCGGCGATAACGTTGGTGTATTGCTTCGTGGTATTGACCGTGACC
AAGTTGTTCGTGGTCAAGTATTGGCTGCTCCAGGCTCAATCCAAACCCACAAGGAATTCAAGGCTCAAGTTTATGTCTTGAAG
AAAGAAGAAGGTGGACGTCACACTCCATTCTTCTCAGACTACCGTCCACAATTCTACTTCCACACCACTGATATTACTGGTGA
GATTGATGCCCAAAAGGGTAA

Conclusion Regarding the Generally Recognized as Safe (GRAS) Status of the Use of the Probiotics *Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, and *Bifidobacterium bifidum* Rosell®-71 Individually or in an 80:10:10 Blend

We, the undersigned members of the Expert Panel, are qualified by scientific education and experience to evaluate the safety of the addition of probiotic bacteria to conventional foods, toddler foods, and infant formulas. We have individually and collectively critically evaluated the materials summarized in the attached monograph and discussed our conclusion among ourselves.

We recognize that *Lactobacillus* and *Bifidobacterium* species have long histories of safe use and are appropriately regarded as non-pathogenic and non-toxicogenic. We conclude that all three bacterial strains have been adequately identified and characterized and that both phenotypic and genotypic research confirm that no concerns exist regarding the safety of ingestion of these probiotic bacteria by infants and children, individually or in a blend of 80% *L. helveticus*, 10% *B. longum* ssp. *infantis*, and 10% *B. bifidum*, at levels up to $5x10^9$ cfu/day. We have applied decision-tree analyses that confirm the safety of each strain and the blend. Therefore, we conclude that addition of the probiotic strains *Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, and *Bifidobacterium bifidum* Rosell®-71, individually or in an 80:10:10 blend, to milk-based infant formula intended for term infants at concentrations consistent with cGMP needed to provide up to $3x10^9$ cfu/serving throughout the shelf life of the product, is safe. We further conclude that the intended use of strains *Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, and *Bifidobacterium bifidum* Rosell®-71, individually or in an 80:10:10 blend, is GRAS based on scientific procedures.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion.

Robert J. Nicolosi, Ph.D.
Professor Emeritus
Health and Clinical Sciences
University of Massachusetts-Lowell

Signature: (b) (6) Date: 17 January 2018

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

John A. Thomas, Ph.D.
Adjunct Professor
Pharmacology & Toxicology
Indiana University Medical School

Signature: _____ Date: _____

Conclusion Regarding the Generally Recognized as Safe (GRAS) Status of the Use of the Probiotics *Lactobacillus helveticus* Rosell®-52, *Bifidobacterium longum* ssp. *infantis* Rosell®-33, and *Bifidobacterium bifidum* Rosell®-71 Individually or in an 80:10:10 Blend

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Robert J. Nicolosi, Ph.D.
Professor Emeritus
Health and Clinical Sciences
University of Massachusetts-Lowell

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Signature: (b) (6) Date: 19 January 2018

John A. Thomas, Ph.D.
Adjunct Professor
Pharmacology & Toxicology
Indiana University Medical School

Signature: _____ Date: _____

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Robert J. Nicolosi, Ph.D.
Professor Emeritus
Health and Clinical Sciences
University of Massachusetts-Lowell

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

John A. Thomas, Ph.D.
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Indiana U[redacted] Medical School

Signature: (b) (6) Date: 1/17/18